JOURNAL OF EXPERIMENTAL BOTANY

AN OFFICIAL ORGAN OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY

Volume 9 *February* 1958 *Number* 25

CONTENTS

F. C. Steward, J. F. Thompson, and J. K. Pollard: Contrasts in the Nitrogenous Composition of Rapidly Growing and Non-growing Plant Tissues 1

F. C. Steward, R. G. S. Bidwell, and E. W. Yemm: Nitrogen Metabolism, Respiration, and Growth of Cultured Plant Tissue 11

S. G. Boatman and W. Mary Crombie: Fat Metabolism in the West African Oil Palm (*Elaeis guineensis*). 2. Fatty Acid Metabolism in the Developing Seedling 52

Clarence Sterling and B. J. Spit: Submicroscopic Structure of the Cereal Starch Grain 75

Leonora Reinhold and R. G. Powell: The Stimulatory Effect of Indole-3-Acetic Acid on the Uptake of Amino-Acids by Tissue of *Helianthus annuus* 82

D. J. D. Nicholas and Tessa Goodman: The Effects of Deficiencies of Zinc and Iron on Some Enzyme Systems in *Neurospora* 97

Kaiser Naguib and Thomas Kennedy Walker: Microbiological Synthesis of Fat 109

L. J. Middleton and R. Scott Russell: The Interaction of Cations in Absorption by Plant Tissues 115

G. E. Briggs, A. B. Hope, and M. G. Pitman: Exchangeable Ions in Beet Disks at Low Temperature 128

G. E. Neal and A. C. Hulme: The Organic Acid Metabolism of Bramley's Seedling Apple Peel 142

UNIVERSITY LIBRARY
29 APR 1958
READING

OXFORD: AT THE CLARENDON PRESS

JOURNAL OF
EXPERIMENTAL BOTANY

Editorial Committee: T. A. BENNET-CLARK (Editor), E. ASHBY, G. E. BLACKMAN, R. BROWN, F. G. GREGORY, W. O. JAMES, W. H. PEARSALL, R. D. PRESTON, M. THOMAS.

THE JOURNAL OF EXPERIMENTAL BOTANY is sponsored by the Society for Experimental Biology as a medium for the publication of papers in the field of plant physiology, biochemistry, biophysics, experimental agronomy, and related topics.

Three numbers comprise a volume and they appear at four-monthly intervals. Subscription price 55*s.* per volume. Single numbers 22*s.* net.

NOTICE TO CONTRIBUTORS

Papers for publication should be addressed to Professor T. A. Bennet-Clark. The right of publication in this journal is not restricted to members of the Society and contributions from research workers both in Great Britain and abroad will be welcomed.

Manuscripts must be typewritten with double spacing. Standard botanical and chemical terminology and abbreviations should be used and contributors are asked to cite references in standard form and present the summary at the beginning rather than at the end of their papers.

Illustrations where possible should be suitable for reproduction as line blocks in the text, and half-tone blocks can also be provided in the text. The maximum size of a printed figure is $7\frac{1}{8} \times 4\frac{1}{8}$ in. Lettering of figures, whether text-figures or plates, should be in pencil. It would be convenient, so as to avoid errors in lettering, if authors would provide a rough duplicate of the lettering of figures. The original figures should be not larger than foolscap size.

Contributors will receive one proof in page. An allowance of 10*s.* per sheet of sixteen pages is made for alterations in the proof, printers' errors excepted, and contributors will be responsible for any excess.

Contributors receive twenty-five copies of offprints free of charge, and may purchase further copies at rates which will be quoted on application to the publisher.

Correspondence should be addressed as follows:

On editorial matters to Professor T. A. Bennet-Clark, Department of Botany, University of London, King's College, Strand, London, W.C. 2.

With regard to membership of the Society for Experimental Biology, to the Hon. Botanical Secretary, Dr. G. E. Fogg, Department of Botany, University College, Gower Street, London, W.C. 1.

Members of the Society not yet receiving the Journal regularly who wish to become subscribers are asked to communicate with the Publications Secretary, c/o The Institute of Biology, 41 Queen's Gate, London, S.W. 7, who will deal with all matters of supply of the Society's journals to members.

On all other matters, to the publishers, Oxford University Press, Amen House, Warwick Square, London, E.C. 4.

Contrasts in the Nitrogenous Composition of Rapidly Growing and Non-growing Plant Tissues

F. C. STEWARD, J. F. THOMPSON,[1] AND J. K. POLLARD

Botany Dept., Cornell University, Ithaca, N.Y., U.S.A.

Received 12 May 1957

SUMMARY

Contrasts have been drawn between the nitrogenous composition of rapidly proliferating tissue and that of the resting, non-growing tissues from which it was derived. The alcohol soluble fraction and the hydrolysate of the alcohol-insoluble fraction of tissue in these contrasted states have been examined by the methods of quantitative partition chromatography.

The ratio of alcohol-soluble to alcohol-insoluble nitrogen was much greater in the resting than in the proliferating tissue. These differences were due mainly to a very much lower content of amino-acids and amides in the growing tissue, especially of asparagine, glutamine, and arginine. Only γ-aminobutyric acid increased in its content in the growing potato cells.

Without regard to the means adopted to induce growth the protein-nitrogen of the dividing cells was conspicuously rich in hydroxy-L-proline, an amino acid, which is conspicuous in collagen but not normally so in plant proteins. The proteins of the growing cells also tend to be rich in the basic amino-acids.

It is suggested that the hydroxyproline-rich protein moiety is a characteristic feature of the proliferating cells and its possible significance is noted.

To understand growth, one should know what enables cells to divide; to understand differentiation, one should understand the circumstances that permit living but mature cells to remain in the plant body without further growth and development. The characteristics of the growing proliferating cell, in which energy and metabolism are canalized toward growth by multiplication of cells and organelle formation, are obviously different from those of living but non-dividing cells, in which the energy of metabolism is not so closely linked to growth and development.

Within the same type of tissue, the transition from the quiescent non-dividing state to the continuously proliferating, undifferentiated, condition may be achieved in different ways, (*a*) by the continuous exogenous supply of special cell-division factors, supplementary to known organic and inorganic nutrients, vitamins or growth factors, or (*b*) by permanent alterations in the metabolism in ways which determine the endogenous supply of factors or principles that promote cell division. The former situation (*a*) is well illustrated by the now familiar examples in which certain natural nutrient fluids, such as the liquid endosperm of coconut milk, or extracts from immature fruits of corn (*Zea*) or of *Aesculus* convert explants of resting tissue to rapidly growing tissue cultures. The latter situation (*b*) is well illustrated by the conversion of

[1] Present address: U.S. Plant, Soil, and Nutrition Laboratory, Ithaca, N.Y.

normal adult tissue to tumorous growth by such stimuli as those of crown gall, or those responsible for tumours of genetic origin. However the growth induction is achieved, the differences between the actively dividing, proliferating cells on the one hand, and non-growing, metabolizing, mature cells on the other, should be determined: As expressed by Rhoades (1949), discussing neoplasms in animals: 'Further data are urgently required on the comparative composition of normal and neoplastic cells. These data may concern genes, enzymes, proteins, or any other component. They may be derived from direct analytical methods, from tracer studies, or from the different susceptibilities of the two cell types to toxic agents.'

The purpose of this paper is to contrast the composition of proliferating cells of actively growing plant tissue cultures with the composition of the cells as they existed in comparable non-growing tissue. The comparisons will be based on the nitrogenous constituents of both types of tissue.

PART I. NORMAL TISSUE IN RELATION TO DERIVED TISSUE CULTURES

(*With* J. F. THOMPSON)

Tissue cultures have been grown from explants of carrot-root phloem by the addition of coconut milk (Steward, Caplin, and Millar, 1952), and from potato cortex by the addition of coconut milk and 2,4-D (Steward and Caplin, 1951). When such growth induction occurs, many changes ensue; the total amount of alcohol soluble nitrogen in the tissue decreases and the alcohol insoluble nitrogen (protein) increases; the water content rises to over 90 per cent.; respiration increases and its sensitivity to enzyme inhibitors changes; and notable differences in the manner of the salt absorption by the two types of tissue have been detected.

Analyses have now been made of such tissue cultures and of comparable tissue from the organ from which these cultures were derived. Samples consisted of tissue from a carrot root or potato tuber before and after the tissue had been induced to grow. Tissues to be analysed were first extracted thoroughly with 70 per cent. ethanol, yielding an alcohol soluble fraction which contained the free amino-acids and amides of the tissue, and a 70 per cent. alcohol insoluble residue which contained the protein. The protein-containing residue was hydrolysed 18 hours in 6N HCl at 120° C., freed of excess HCl by evaporation, and neutralized to pH 7·0 for subsequent paper chromatography.

All amino-acid analyses were made using a quantitative method previously described (Thompson and Steward, 1951). This method gives reliable results for the amino-acids to which it is applicable including those present in typical protein hydrolysates; cysteine, histidine, and tryptophan require other procedures. Prior to chromatography the total amount of ninhydrin reactive substances in the extracts and protein hydrolysates was determined by the method of Moore and Stein (1948).

The data of Table I show that both the non-protein nitrogen content and the ratio of non-protein to protein nitrogen decrease with the onset of growth. This reflects the active protein synthesis which is a conspicuous feature of the growth induction. In spite of the decrease in per cent. dry weight which occurs

TABLE I

Characteristics of Tissue Analysed

Characteristic	Carrot		Potato	
	Initial non-growing	Final growing	Initial non-growing	Final growing
Fresh weight mg./explant	3·0	107·8	3·0	45·7
Non-protein nitrogen μg./g. fresh weight	801	124	1,281	452
Protein nitrogen μg./g. fresh weight	741	898	1,083	2,200
Non-protein nitrogen μg./explant	2·4	13·4	3·8	20·6
Protein nitrogen μg./explant	2·2	96·6	3·2	100
Ratio of non-protein to protein nitrogen	1·07	0·138	1·18	0·205
Protein nitrogen synthesized μg./explant	94·7		97·2	
Ratio of final to initial protein nitrogen	44		31	

with the initiation of growth, protein content (protein-N per unit fresh weight) as well as total protein per culture increase, indicating that protein synthesis has outpaced growth and has also outpaced the formation of other constituents.

The amino-acid analyses of both the protein and non-protein fractions (Table II) show that both qualitative and quantitative changes in these fractions accompanied the 30- to 40-fold increase in protein nitrogen per explant, and the concurrent 5- to 6-fold increase in the non-protein nitrogen per explant which are recorded in Table I. The data in Table II indicate that in the non-growing tissue the general composition of the total protein of carrot and potato was somewhat different as might well be expected, notably the potato protein was richer in the basic amino-acids. But the change from the non-dividing to the dividing state was accompanied in both cases by changes in the composition of the protein synthesized. The outstanding quantitative change which was common to both carrot and potato tissue is the relative increase in hydroxyproline content of their protein. The increase in hydroxyproline in the proteins of the growing cell was especially dramatic in the case of potato, since this amino-acid was not chromatographically detectable in the resting potato tuber. In the case of the carrot, however, the proline and hydroxyproline content of the dividing cells was more than double that of the resting cells.

The non-protein amino-acid content (Table III) of the growing tissue was much less than that of the non-growing tissue. In tissue from the carrot root

TABLE II

Comparison of the Amino-acid Composition of the Bulk Proteins of Carrot Root and Potato Tuber with that of Tissue Cultures derived from them

(All data represent amino-acid nitrogen as percentage of total protein nitrogen)

Amino-acid	Potato tuber (variety Katahdin)		Carrot root	
	Original tissue	Active tissue culture	Original tissue	Active tissue culture
Aspartic	9·2	7·0	9·6	9·3
Glutamic	10·2	8·0	11·5	9·0
Glycine	7·6	6·7	8·9	7·7
Serine	3·6	5·5	5·5	5·2
Threonine	2·6	3·2	3·4	4·7
Alanine	7·1	6·6	7·6	8·0
Lysine	11·2	13·6	6·1	5·5
Arginine	14·0	11·8	5·0	7·0
Methionine	1·0	1·4	0·56	0·81
Proline	3·1	3·9	3·4	7·1
Valine	6·7	5·8	8·1	7·8
Leucines	13·6	12·0	18·2	13·5
Phenylalanine	7·8	5·8	5·9	2·8
Tyrosine	2·2	4·0	2·9	2·5
Hydroxyproline	0·0	4·8	3·6	9·2

TABLE III

Comparison of the Nitrogenous Composition of the Alcohol-soluble Fraction of Mature, Non-growing and Tissue-cultured Plant Tissues

(μg. N per amino-acid per gm. fresh weight)

	Potato tissue		Carrot tissue	
	Non-growing	Cultured	Non-growing	Cultured
Aspartic acid	11·22	1·44	25·9	1·2
Glutamic acid	16·95	12·73	42·0	4·5
Serine	8·81	7·28	17·0	1·5
Glycine	5·15	8·00	1·7	0·4
Asparagine	291·5	0·0	153·3	0·0
Threonine	11·83	4·50	11·8	0·7
Alanine	20·75	21·50	93·0	10·4
Glutamine	579·0	15·72	77·8	0·0
Lysine	12·1	2·62	0·5	0·0
Arginine	114·5	15·46	35·2	0·0
Methionine	7·74	0·0	2·61	0·0
Proline	0·0	0·0	4·2	0·1
Valine	29·1	6·31	8·6	0·9
Leucines	11·15	8·05	7·8	0·7
Phenylalanine	11·71	6·18	13·3	0·0
Tyrosine	9·38	3·66	1·6	0·0
γ-Aminobutyric acid	40·75	88·3	18·4	2·3

a striking decrease in the level of all the free amino-acids accompanied the growth induction. In the case of potato tissue, while most of the free amino-acids decreased, several showed little change whereas γ-aminobutyric acid actually increased in concentration under conditions which induced rapid growth.

To determine whether the observed differences between normal and cultured tissue of carrot and potato were confined to some specific influence of coconut milk, or whether they were more generally representative of differences between rapidly growing and non-growing cells, certain other analyses were made on two different tissues that exhibit rapid, undifferentiated growth in response to causal agents other than coconut milk. These contrasts were made by analysing normal tissue and the genetic tumours of *Nicotiana* and both normal and crown gall tissue of *Kalanchöe*.

PART II. PLANT TUMOURS IN RELATION TO NORMAL TISSUE

(*With* J. F. POLLARD)

In the case of *Kalanchöe*, young plants were inoculated with the crown gall organism, *Agrobacterium tumefaciens*, and the tumours allowed to develop until they eventually became sterile. When sufficient material was available the tumours were harvested and analysed, using the methods described above.

TABLE IV

A Comparison of the Amino-acid Composition of the Bulk Protein of Normal and Crown-gall Infected Leaves of Kalanchöe daigremontiana

	Micrograms N in each amino-acid per g.f.w.			Per cent. of total N accounted for by each amino-acid		
Amino-acid	Normal leaves	Tumour-bearing leaves	Leaf tumours	Normal leaves	Tumour-bearing leaves	Leaf tumours
Aspartic . .	74·9	54·1	114·7	9·79	8·07	9·25
Glutamic . .	55·6	52·6	107·5	7·27	7·85	8·67
Serine . . .	46·9	31·2	63·4	6·13	4·66	5·11
Glycine . .	67·7	54·8	111·3	8·85	8·18	9·12
Threonine . .	48·2	42·5	77·1	6·30	6·34	6·22
Alanine . .	76·3	54·1	102·5	9·97	8·07	8·27
Lysine . . .	90·0	67·4	89·7	11·70	10·06	7·24
Arginine . .	132·0	107·0	143·1	17·25	15·96	11·55
Proline . . .	25·4	82·6	138·8	3·32	12·32	11·20
Valine . . .	55·6	41·0	98·8	7·27	6·12	7·97
Leucines . .	84·5	82·9	167·5	11·04	12·37	13·51
Tyrosine . .	7·8	none	11·9	1·01	0·00	0·96
Hydroxyproline .	none	none	13·1	0·00	0·00	1·06
Totals . .	765	670	1,240	—	—	—

The composition of the protein in the leaf tumours was compared in this way with the composition of the protein of both leaves from uninfected plants and

the otherwise normal appearing parts of leaves which bore tumours. These data appear in Table IV.

The tumours were considerably richer in protein than the normal leaves or the normal parts of tumour-bearing leaves. Furthermore hydroxyproline was again a feature of the tumour protein. Although it only represented about 1 per cent. of the protein nitrogen in the tumours, hydroxyproline was undetected in the unaffected tissues.

Dr. H. H. Smith, of the Cornell Department of Plant Breeding, supplied plants of a tobacco hybrid (*Nicotiana glauca* × *N. Langsdorffii*), which is well

TABLE V

Amino-acid Composition of the Bulk Proteins of some Tobacco Tissues

	Micrograms of amino-acids per g.f.wt.				Nitrogen of each amino-acid as percentage of total nitrogen determined chromatographically			
Amino-acid	*Nicotiana Langsdorffii* leaves	*N. glauca* leaves	*N. glauca* × *N. Langsdorffii* leaves	*N. glauca* × *N. Langsdorffii* tumours	*Nicotiana Langsdorffii* leaves	*N. glauca* leaves	*N. glauca* × *N. Langsdorffii* leaves	*N. glauca* × *N. Langsdorffii* tumours
Aspartic acid	135·0	256·0	141·0	150·0	6·0	7·8	6·1	6·0
Glutamic	188·0	302·0	188·0	143·0	8·4	9·3	8·1	5·7
Serine	114·0	108·0	145·0	110·0	5·1	3·3	6·3	4·4
Glycine	226·0	327·0	282·0	252·0	10·0	10·0	12·2	10·1
Threonine	91·3	116·0	109·0	89·1	4·1	3·6	4·8	3·6
Alanine	189·0	261·0	208·0	151·0	8·4	8·0	9·0	6·0
Histidine	Present	Present	Present	Present	Present	Present	Present	Present
Lysine	201·0	535·0	324·0	326·0	9·0	16·4	14·0	13·0
Arginine	324·0	530·0	350·0	477·0	14·5	16·2	15·1	19·0
Methionine	Present	Present	Present	Present	Present	Present	Present	Present
Proline	214·0	125·0	188·0	152·0	9·5	3·8	8·1	6·1
Valine	116·0	108·0	120·0	94·8	5·2	3·3	5·2	3·8
Leucine	185·0	160·0	150·0	197·0	8·2	4·9	6·5	7·8
Phenylalanine	242·0	382·0	36·0	236·0	10·8	11·7	1·6	9·4
Tyrosine	18·2	57·2	67·0	47·7	0·8	1·7	2·9	1·9
Hydroxyproline	None	None	Trace	79·9	0·0	0·0	0·0	3·2
Total	2,244	3,267	2,308	2,935	—	—	—	—

known to develop tumours spontaneously, and also plants from both the parental stocks. Analyses were made as above on the tumours, the otherwise normal parts of the tumour-bearing hybrid, and on tissue from both parents, neither of which develops tumours spontaneously; these data are summarized in Table V. Measurable amounts of hydroxyproline were found only in the tumours.

GENERAL DISCUSSION

Properties of rapidly dividing cells of plant-tissue cultures which contrast with those of resting or non-dividing cells may now be summarized as follows: (*a*) Markedly increased water content of the dividing cells; (*b*) greatly increased respiration which carries with it a stimulus to the absorption of ions (Steward and Millar, 1954); (*c*) changes in the respiratory pathways as indicated by the different sensitivity of the growing and the non-growing tissues to enzyme inhibitors (Steward and Millar, 1954); (*d*) marked reduction in the alcohol-soluble nitrogen content of the tissue in relation to its alcohol insoluble, or protein–nitrogen counterpart. In other studies it has also been shown (Steward and Shantz, 1955) that potato-tuber cells stimulated to divide by coconut milk

tend to remain small, their average size approximating 0·16 μg. per cell, as contrasted with the average size of the cells of the original explants (1·4 μg. per cell). In the present study further contrasts have been drawn between the composition of the relatively mature parenchyma cells, the further growth of which is predominantly by cell expansion, and that of rapidly dividing, more meristematic, cells from the same tissue. The main points of difference that have arisen from this study can be summarized as follows.

First, there is the obvious point that the relative composition of the soluble nitrogen fraction in the growing, protein-synthesizing cells in no way reflects the relative amino-acid composition of the protein which is synthesized. This implies that the synthesis involves something much more than the mere condensation of free amino-acids, as they exist preformed in the tissue (see Tables II and III). On transfer from the mature, non-growing state to the actively proliferating state, however, certain prominent changes in the composition of the soluble nitrogen fraction appeared. The conspicuous changes concerned the amides (asparagine and glutamine) and arginine, all of which are prominent as storage compounds in plants. In particular, asparagine virtually disappeared from the cultured tissue of potato, although it was initially high in the cells of the tuber; the same was also true of tissue cultured from the carrot root. Glutamine, which is also a very rich storage form of nitrogen in the potato tuber, also declined drastically in amount though it persisted in the growing-tissue cultures derived from the potato tuber. However, in the carrot-tissue cultures, in which the total alcohol-soluble nitrogen fraction was reduced to a very low level, glutamine was undetectable. The same trend toward a much lower content of arginine in the growing cultures was also noted. All these comparisons can be seen in Table III. However, in the case of tissue of the potato tuber the transfer from the resting to the growing state was accompanied by an actual enrichment in the content of γ-aminobutyric acid. This is the only notable example of a free amino-acid which increased in the proliferating tissue in comparison with the content of the same compound in the resting, or dormant, cells. Alanine was conspicuous amongst the soluble nitrogen compounds which persisted in the growing-tissue cultures, both in the case of potato and carrot cultures.

The most conspicuous feature of the data as reported, however, is the following. Although different means have been used to induce growth in the otherwise adult cells, hydroxyproline consistently appeared as a more conspicuous amino-acid in the protein of the proliferating cells than in the protein of the cells from which the tissue culture was derived. The different means which have been used to induce growth were (*a*) the use of the growth factors which are present in coconut milk (supplemented, in the case of the potato tissue, by the synergistic effect of 2:4-D or some similar substance); (*b*) the stimulus to cell division which is imparted by the crown-gall organism to the stem or leaf of *Kalanchöe*; and finally (*c*) the effect of the genetic constitution as illustrated by spontaneous tumour formation in *Nicotiana* hybrids. Table II shows that hydroxyproline was not normally present in potato-tuber

protein at a level detectable chromatographically; whereas in the protein of the actively growing tissue substantial amounts appeared. Carrot tissue initially contained some hydroxyproline in its protein, but the hydroxyproline content of the protein increased threefold when the carrot-tissue explants were induced to grow. Hydroxyproline in the protein of *Kalanchöe* occurred only in the tumours which are readily induced on leaves. Although the actual content of hydroxyproline in the total protein of these various tissues was small, its presence is nevertheless highly significant. In the case of tobacco the hydroxyproline only appeared appreciably in the protein of the tumours which formed spontaneously on the hybrid; it did not appear in the protein of the normal tissue of either parent, and only a trace was detectable in the otherwise normal tissue of the hybrid. These data can be seen in Tables II, IV, and V.

The consistent presence of hydroxyproline in the protein of cells which undergo random proliferation, of which four distinct examples are here shown, indicates that protein containing this amino-acid in appreciable quantity must in some way be a characteristic of cells in the randomly dividing state. The analysis of the total protein in the various tissues does show differences, but these figures are inevitably an average of all the proteins which are there present; and, if the hydroxyproline-containing moieties from each type of tissue could be separated and analysed, they might well prove to be more uniform than the data indicate.

These results are the more remarkable because free hydroxyproline is certainly of very rare occurrence in plants, and it never occurs in appreciable amounts. The conspicuous example of the occurrence of hydroxyproline in the free state in plants is that due to Radhakrishnan and Giri (1954), who found this amino-acid in *Santalum*, however, showing it to be allo-hydroxyproline. The hydroxyproline in the protein of plants is presumed to be hydroxy-L-proline, as in collagen. In fact, the hydroxyproline from the hydrolysate of cultured carrot tissue which was supplied from this laboratory, was examined chromatographically by Dr. B. Witkop[1] and definitely shown to be distinct from the allo form. Therefore, this amino-acid (hydroxy-L-proline), which is found to be so conspicuous a feature of the protein of the dividing cells, definitely does not occur in the free state. This raises the question of the biogenesis of hydroxyproline: a problem which will be discussed in a later paper.

In other work from this laboratory various C^{14}-labelled substrates were furnished to tissue cultures which were stimulated to grow with coconut milk, and the movement of the C^{14} into the protein was traced. It was found, perhaps somewhat unexpectedly, that hydroxyproline was one of the most prominently labelled of the protein amino-acids, especially when C^{14}-labelled sugar was furnished. Furthermore, the consideration of the specific activities of the labelled amino-acids led to the view that the protein moiety that contained the hydroxyproline in these cells was not reused in metabolism. These ideas were published in summary form (Steward, Bidwell, and Yemm, 1956) and the evidence will appear more extensively in other papers now in press.

[1] Dr. B. Witkop, National Institutes of Health, Bethesda, Md.

These results raise interesting questions. The conspicuous example of the occurrence of hydroxyproline in nature is its occurrence in collagen, the elastic protein of the animal body. Moreover, it is also known that in various situations in the animal body, in which cells return to the growing state (as in wound healing) collagen synthesis is markedly accentuated (Asboe-Hansen, 1954). Associating these results might suggest that the hydroxyproline-containing moiety in the dividing cells of the plant might well be of a somewhat similar sort to collagen. The interesting question is whether this plant protein may also be an elastic structural element of plant cytoplasm, possibly associated with the elastic elements that may be required in the mitotic figures of the dividing cells and perhaps even contained in the spindle fibres. However, this hydroxyproline-containing protein is apparently more accentuated in randomly proliferating tissues than it is in meristematic cells in the organized growing point, for the examinations which have as yet been made (Steward, Wetmore, Thompson, and Nitsch, 1954) have not revealed this amino-acid in the protein of the meristem, even though it could be detected (in *Lupinus*) in some of the older tissues. It is of course well known that proline occurs universally in protein and it is now conceived that it may fulfil some special function in the configuration of spirally arranged proteins, because the configuration of its molecule permits the protein chain to change direction by turning back upon itself (Edsall, 1954). Conceivably hydroxyproline could function in a similar way, with the inherent possibility of added properties due to the hydrogen bonding which the hydroxyl group would permit. Essentially, however, the true significance of the presence of hydroxyproline in the protein of rapidly proliferating cells, which is here recorded, still remains to be determined.

ACKNOWLEDGEMENTS

This work formed part of a programme of research being directed by the senior author, and supported by grants from the National Cancer Institute of the U.S. Department of Health Education and Welfare. This help is gratefully acknowledged. The work involving aseptic culture of plant tissues was done with the help of Miss K. Mears, research assistant to one of us (F. C. S.).

LITERATURE CITED

Asboe-Hansen (1954). *Connective tissue in Health and Disease.* Munksgaard, Copenhagen, Denmark.

Edsall, J. T. (1954). The configuration of certain protein molecules. An inquiry concerning the present status of our knowledge. *J. Polymer Sci.* **12**, 253.

Moore, S., and Stein, W. H. (1948). The colorimetric method. *J. Biol. Chem.* **176**, 367–88.

Radhakrishnan, A. N., and Giri, D. V. (1954). The isolation of allohydroxy-L-proline from Sandal (*Santalum album L.*). *Biochem. J.* **58**, 57–61.

Rhoades, C. P. (1949). Neoplastic abnormal growth, in *Chemistry and Physiology of Growth*, p. 257. Princeton University Press.

Steward, F. C., Bidwell, R. G. S., and Yemm, E. W. (1956). Protein metabolism, respiration and growth: A synthesis of results from the use of C^{14}-labelled substrates and tissue cultures. *Nature*, **178**, 734–8; 789–92.

—— and Caplin, S. M. (1951). A tissue culture from potato tuber. The synergistic action of 2,4-D and of coconut milk. *Science*, **113**, 518–20.

STEWARD, F. C., CAPLIN, S. M., and MILLAR, F. K. (1952). Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition, and growth in undifferentiated cells. *Ann. Bot.* **16**, 57–77.

—— and MILLAR, F. K. (1954). Salt accumulation in plants: A reconsideration of the role of growth and metabolism. A. Salt accumulation as a cellular phenomenon. *Symp. Soc. Exptl. Biol.* **8**, 367–93.

—— and SHANTZ, E. M. (1955). The chemical induction of growth in plant tissue cultures. Part I. Methods of tissue culture and analysis of growth. From *The Chemistry and Mode of Action of Plant Growth Substances*. Edited by R. L. Wain and F. Wightman, Butterworth Scientific Publications, London, England.

—— WETMORE, R. H., THOMPSON, J. F., and NITSCH, J. P. (1954). A quantitative chromatographic study of nitrogenous components of shoot apices. *Amer. J. Bot.* **41**, 123–34.

THOMPSON, J. F., and STEWARD, F. C. (1951). Investigations on nitrogen compounds and nitrogen metabolism in plants. II. Variables in two-directional paper chromatography of nitrogen compounds: A quantitative procedure. *Plant Physiol.* **26**, 421–40.

Nitrogen Metabolism, Respiration, and Growth of Cultured Plant Tissue

PART I. EXPERIMENTAL DESIGN, TECHNIQUES, AND RECORDED DATA

PART II. THE INTERPRETATION OF SPECIFIC ACTIVITY DATA IN TRACER EXPERIMENTS

PART III. NITROGEN METABOLISM AND RESPIRATION OF CARROT TISSUE EXPLANTS AS REVEALED BY EXPERIMENTS WITH C^{14}-LABELLED SUBSTRATES

F. C. STEWARD
Department of Botany, Cornell University, Ithaca, N.Y.
R. G. S. BIDWELL[1]
Department of Biology, Queen's University, Kingston, Ontario
E. W. YEMM
Department of Botany, University of Bristol

WITH ONE PLATE
Received 12 May 1957

I. EXPERIMENTAL DESIGN, TECHNIQUES, AND RECORDED DATA

SUMMARY

Part I. The effects of growth on the metabolism of undifferentiated plant tissue have been studied, using radioactive substrates, chromatographic techniques, and tissue-culture methods.

Carrot-phloem explants were maintained either in a state of active growth by cell division by adding coconut milk to the basal medium, or in relative quiescence with little growth by withholding it. C^{14}-labelled glucose, glutamine, or γ-aminobutyric acid was added to slow- or fast-growing cultures and the respiration, fresh weight, soluble and insoluble amino-acids and sugars were examined at approximately daily intervals.

From quantitative analyses, the specific activities of respired carbon dioxide, soluble and insoluble amino-acids and sugars were determined, and the amounts and specific activities of carbon in transit into or out of each metabolite in a given unit of original tissue (10 explants) were calculated. All the data are arranged in tabular form for interpretation in this and subsequent papers.

Part II. Certain anomalies in the specific activity of carbon in various metabolites, and particularly in the data for carbon in transit to or from metabolites occur. These anomalies are explained on the basis of the heterogeneity of the cell, in

[1] Present address: Atlantic Regional Laboratory of the National Research Council of Canada, Halifax, Nova Scotia.

terms of separate phases or compartments in the cell. Distinct pools of metabolites subject to different reactions occur in these phases but, on extraction, they mingle and cannot be distinguished. Type situations which interpret the data are presented.

The need for critical examination of specific activity data is stressed since, by appropriate analysis, it may reveal the trend of events in the separate phases.

Part III. The nitrogen metabolism and respiration of carrot tissue explants have been examined, using the data derived from the experiment described in Part I and the analytical methods outlined in Part II.

The important points in the analysis are as follows: (i) The cell is divided into phases or compartments; one associated with assimilative metabolism and protein synthesis, and the other with protein breakdown, catabolism, and storage. (ii) There are two main sources of carbon dioxide in the cell, one arising by the direct oxidation of sugars, and the other from the oxidation of breakdown products arising from protein turnover. (iii) Free amino-acids of the tissues are not the immediate precursors of protein. Protein is formed mainly from carbon skeletons derived from glucose and nitrogen groups which are combined and immediately converted to protein at a site remote from the soluble amino-acids.

Interrelationships between various nitrogenous compounds are discussed. In particular, it is evident that γ-aminobutyric acid, instead of being a decarboxylation product of glutamine or glutamic acid, is an important precursor of these compounds, and hence has a much more central role in the nitrogen metabolism of these tissues than had been thought. There is a striking relationship between the C^{14} of glutamine and γ-aminobutyric acid and free proline and proline and hydroxyproline combined in the protein. These and other observations suggest that certain hydroxyaminoacids may be formed *in situ* in the protein.

INTRODUCTION

WHEREAS much attention has been directed to the interpretation of intermediary carbohydrate metabolism and respiration by the use of radioactive tracer techniques, the same degree of attention has not been given to nitrogen metabolism and the relation of nitrogen compounds to respiration and protein synthesis. Similarly, little work has been done utilizing homogeneous or unspecialized plant-tissue systems contrasting relatively inactive mature cells with those which are in a state of active growth. A very broad plan showing the inter-relationships of the various metabolites and processes involved, also the general background for further more detailed investigations, has been designed. A preliminary account of this work has already appeared in *Nature* (Steward, Bidwell, and Yemm, 1956).

The plan of the investigation was as follows. The plant tissue selected was the secondary phloem obtained from the carrot root. This tissue is readily grown in sterile tissue culture and has the particular advantage that it responds to certain growth factors which enable it to proliferate rapidly, or, in the absence of these substances, it remains in a more or less quiescent condition. Moreover, the procedures necessary to achieve these ends were already familiar (Steward, Caplin, and Millar, 1952). The C^{14} was supplied to this tissue in the form of small amounts of uniformly labelled γ-aminobutyric acid, glutamine, or glucose. These compounds were furnished to the tissue under such conditions that they acted as tracers rather than as nutrients—the

purpose being to mark the pathways characteristic of the growing and non-growing tissue respectively, and not to compel the tissue to depart from its normal metabolism. Whereas the two labelled nitrogen compounds intervene in the nitrogen metabolism, the glucose intervenes in the carbohydrate metabolism; and by the combined techniques of chromatography and radio-autography their ramified effects could be followed. These objectives were obtained by the following operations:

1. Carrot-tissue cultures were grown under conditions previously described (Steward, *et al.*, 1952) with, and without, the coconut-milk growth factors for a pre-period of 7 days. This allowed the tissue to become adjusted to the particular culture conditions to which it was exposed.

2. At this point one or other of the C^{14}-labelled substances was added to the external medium.

3. From this time the tissues were sampled during 5 days, extracts of the alcohol-soluble fractions were obtained and the alcohol-insoluble residues were retained for subsequent hydrolysis.

4. The alcohol-soluble compounds and the acid hydrolysates of the alcohol-insoluble material were examined by standard techniques of paper chromatography and radioautography with the primary emphasis on the substances that react with ninhydrin. The distribution of the C^{14} amongst the different compounds so detected was recorded and quantitative determinations were made showing the amount of these substances present and the amount of radioactivity that they contained.

5. From these data specific activities were calculated for the substances as they existed in the tissue. At the same time-intervals the respired carbon dioxide was collected and determined, its radioactivity measured, and its specific activity calculated.

6. Fresh weights of the sampled tissues were obtained. Total amounts and changes in the various metabolites during the course of the experiment and in the radioactivity they contained were calculated.

EXPERIMENTAL TECHNIQUES

The tissue and tissue-culture conditions. To adapt the tissue-culture methods for the special purposes of the investigation, the following changes were made.

White's (1943) basal medium was used. Coconut milk was added where the tissue was required to be in the actively proliferating state, but its concentration was reduced to 2 per cent. of whole coconut milk by volume. Glucose was used as the only sugar in the medium, instead of sucrose, at a lower concentration, 0·5 per cent. for the slower-growing tissue and 1 per cent. for the more actively growing tissues. These conditions represented a compromise between supplying the amount of sugar that would achieve the maximum amount of growth and the need to avoid excessive dilution of the radioactive sugar by additional carbohydrate. To obtain enough

tissue for analysis, the number of carrot explants per culture tube was increased to 10 for the slow-growing tissue and 5 per tube for the rapidly growing cultures. Although this use of multiple explants increases the risk of infection, special precautions were taken and the whole experiment was carried out aseptically without any contaminations. Although these conditions involved the use of a *reduced* concentration of sugar and an *increased* amount of tissue in the culture tubes, at no time was the sugar supply exhausted, the final sugar concentration being approximately 70 per cent. of the original concentration. Similarly the radioactive nitrogen compounds were not more than half exhausted even by the actively growing tissue.

The form of the growth curve for single cultures under these conditions has already been described; the use of replicate cultures in each tube does not appreciably alter this growth pattern.

The radioactive substances used. All the radioactive substances applied as tracers were uniformly labelled and arose from biological syntheses carried out in the laboratory of Dr. G. Krotkov at Queen's University, Kingston, Ontario. The C^{14}-labelled glucose was obtained by Dr. P. V. Vittorio from tobacco leaves (Vittorio, 1953) and had a specific activity of 2×10^6 counts per min. per mg. of carbon. The C^{14}-labelled glutamine was obtained from swiss chard leaves by one of us (R. G. S. B.) by the method of Bidwell, Krotkov, and Reed (1954), and the specific activity of its carbon was 890,000 counts per min. per mg. This glutamine contained a small impurity, not exceeding 4 to 5 per cent., of pyrrolidone carboxylic acid. From the C^{14}-labelled glutamine a sample of C^{14}-labelled γ-aminobutyric acid was prepared by one of us (E. W. Y). This conversion was achieved by treating a solution of the glutamine with a decarboxylase obtained from *Clostridium Welchii*, used under conditions described by Yemm and Folkes (1954). After the enzymic reaction was complete γ-aminobutyric acid was isolated by the use of a copper-carbonate technique (Thompson, Pollard, Steward, 1953), combined with treatment with ion-exchange resin (Amberlite XE64) to remove the traces of copper from the effluent. The resultant γ-aminobutyric acid was shown to be chromatographically pure and the yield obtained approached the theoretical amount. The specific activity of the carbon in the γ-aminobutyric acid was exactly the same as that of the carbon in the glutamine from which it was derived.

Whereas the solutions of C^{14}-labelled glucose and γ-aminobutyric acid which were added to the cultures were sterilized by heat, the glutamine solution was sterilized by passing it through a sintered-glass bacteriological filter. Aliquots of the sterile stock solutions were then added to the appropriate culture tubes after growth had proceeded for 7 days.

The conditions of the experiment. A prominent feature of the experiment was the contrast between the slowly growing carrot explants, in the absence of the coconut milk, and the very much more rapidly growing explants in the presence of the coconut milk. Although there were 10 explants per culture tube in the slow-growing cultures and 5 in the fast-growing, all the data in

the tables are calculated to a standard unit consisting of 10 carrot explants. This is done for the sake of uniformity and to enable direct comparisons to be made on the tissue-culture basis. Since the primary interest of the investigation concerns the total changes that occur as a result of the treatments, the data have not been presented on the tissue-weight basis but rather on the unit-culture basis. As a further consequence of the contrasted conditions employed, it was necessary to ensure that the more rapidly growing tissue did not exhaust the radioactive compounds supplied. For this reason larger total quantities of the substances were added to the cultures containing the rapidly growing tissue with coconut milk. The amounts of the C^{14}-labelled compounds added per 10 explants are given in Table I.

TABLE I

Radioactive compounds supplied per 10 carrot explants grown with or without coconut milk (C.M.)

Compound	Slow growing cultures (No C.M.)		Fast growing cultures (+C.M.)	
	mg.	c./min. $\times 10^{-3}$	mg.	c./min. $\times 10^{-3}$
C^{14}-glucose . . .	1·025	823	4·10	3,308
C^{14}-γ-aminobutyric acid	1·935	802	7·74	3,210
C^{14}-glutamine . .	1·970	805	7·88	3,300

The amounts of the C^{14}-labelled substances to be added to the culture solution were decided as follows. Since the approximate amount of these compounds in the tissue were known, they were supplied at a level such that they would label the metabolic pool sufficiently for identification and assay of radioactivity.

The following comments are necessary on the time intervals adopted. The tissue was grown under the two types of culture conditions for 7 days prior to the addition of the radioactive substances. The end of this preliminary period, when the radioactive substances were added to the culture solutions, is therefore designated Time 0. Subsequent sampling periods, at approximately 24-hour intervals, are designated in the tables by the numbers 1 to 5. The complete measurements and samples (fresh weight of the tissue, respiration, uptake and loss of radioactivity, samples for the determination of nitrogen constituents and carbohydrate constituents of the tissue) were all obtained at periods 1, 2, 3, and 5. At period 4, however, only carbon dioxide determinations were made.

Measurement of respiration. The methods of Steward, Caplin, and Millar (1952) were used for collection and determination of respired carbon dioxide. For the special purposes of this investigation this technique was modified as follows. Each culture tube was fitted with a standard tapered joint on its side neck to which a vessel containing alkali was attached. While several types of CO_2-collecting vessels were tried, the most useful one consisted of a second culture tube, which contained a suitable amount of caustic soda, and was attached by a standard taper ground glass joint. The culture tubes with their

carbon dioxide collecting reservoirs attached were placed on the revolving shaft, as in the normal growth of the cultures. At the end of the appropriate periods a known volume of carefully standardized sodium carbonate solution was added to each collecting tube. This was done to dilute the radioactive carbon dioxide respired so that it could be more readily counted. Barium chloride was then added to the carbon dioxide collecting tube and the excess alkali was titrated to phenolphthalein using standard hydrochloric acid and a microburette. The titration was done in the collecting tubes and it was found that the replicate determinations were in very close agreement.

The respiration determinations were carried out immediately in the Cornell laboratory and the precipitated barium carbonate was used for subsequent determinations of the specific activity of the respired carbon dioxide at Kingston. The barium carbonate from replicate cultures was pooled and the specific activity of the carbon it contained was determined by a method which has been described (Calvin *et al.*, 1949). From all these data the total amount of carbon dioxide respired in a given period from a unit-tissue culture was calculated. It was feared that the values for total evolved carbon dioxide and its total radioactivity might be low due to its retention in the culture medium. However, the addition of strong acid to the media caused neither significant loss of activity from the media nor the evolution of detectable amounts of carbon dioxide or other radioactive volatile substances.

The final external solutions. The amount of radioactivity added to the external solution and the amount remaining at the end of each sampling period was determined. Thus, the amount of C^{14}-labelled substance absorbed by the tissue was estimated. In the case of γ-aminobutyric acid and glutamine, the C^{14}-labelled substance added was all that the medium contained of these substances. Therefore, the specific activity of these compounds in the medium was the same as that of the compound added. In the case of glucose, however, an unknown amount of non-radioactive glucose was present when the radioactive substance was added. Consequently an aliquot of this solution was withdrawn to determine the specific activity of glucose. This was done by chromatographing an aliquot of the external solution in one direction using butanol-ethanol-water (45:5:50) (Partridge, 1946). Glucose was the only radioactive substance on these chromatograms, and its specific activity was determined as described later.

The final treatment of the tissue. At each sampling period the tissue cultures from replicate tubes were pooled. After obtaining their fresh weight in milligrams the tissues were extracted in three successive changes of cold 70 per cent. ethanol and finally with hot 50 per cent. ethanol. The combined extracts were evaporated to dryness in a stream of air. The extracted tissue residues were then treated as follows:

1. They were hydrolysed with 10 ml. of 3 N HCl at 120° C. for 6 hours.
2. The acid humin was removed, washed, and the washings and the hydrolysate were combined and evaporated to dryness. The evaporation was repeated to remove excess of HCl. The hydrolysate was finally adjusted

to pH 5–6 (bromophenol blue) with an appropriate volume of 2N NaOH. The alcohol extracts of the soluble compounds and the acid hydrolysates of the alcohol-insoluble compounds were retained for chromatographic analysis by methods which are described below.

Chromatographic techniques. Consideration was given to the appropriate solvent systems for use in the two-directional paper chromatography of the extracts obtained as above. The solvent system finally chosen was that most suitable for the detection of both the amino-acids and the sugars which were present. Phenol: collidine-lutidine was most convenient because it placed the sugars in a part of the chromatogram which was completely free from ninhydrin reactive compounds. (By the use of the alternative phenol: butanol-acetic acid system the sugars were found in positions too close to some of the amino-acids.) Under the procedure thus adopted fructose was clearly separated as a single compound whereas sucrose and glucose superimposed. Thus, the amount and activity of fructose were unambiguously obtained, whereas glucose and sucrose were determined as a combined sample. The amount of each extract on the chromatogram was adjusted to provide good resolution of the various compounds and enough material for the quantitative determinations, namely 50 μg. nitrogen per paper. Duplicate chromatograms of each extract were made in the same cabinet. Using one of these, sprayed with ninhydrin, the identity of the substances present was determined, a tracing was kept for subsequent record, and the quantities of each of the identified constituents were determined (Thompson and Steward, 1951). Special steps ensured the calibration curves were accurate for the smaller amounts determined. The remaining chromatogram of each sample, *not* sprayed with ninhydrin, was radioautographed using Ansco no-screen X-ray film, the exposure time being predetermined from the activity present. Representative radioautographs are shown in Fig. 2 (Plate I).

Radioactive techniques. Where possible spots on the radioautographs were associated with the ninhydrin reactive spots, and areas of radioactivity not associated with ninhydrin activity were denoted by a suitable code of numbers (R1–R12). Radioactive areas were traced from the radioautograph on to the unsprayed chromatogram, thus permitting areas of activity to be located and cut out so that the radioactive compounds could be eluted. The radioactive compounds were eluted in 3 to 5 ml. of water and aliquots (100 to 200 μl.) of the eluate were plated and their activities determined in a methane gas-flow counter. Using this counter, tests have shown (Nelson and Krotkov, 1955) that the same results are obtained by counting either organic carbon or the carbon dioxide, as $BaC^{14}O_3$, derived from its oxidation. All the activities recorded refer to data obtained directly from the counter which operated with approximately 50 per cent. efficiency, and they are not corrected to absolute values.

The specific activity of the ninhydrin reactive compounds was determined from the radioactivity and the total amount of the compounds as determined by the ninhydrin method.

Determination of sugars. Each spot from the chromatograms which contained sugar was eluted with 4 ml. of water, 2 ml. of this eluate were placed in a small test-tube and 4 ml. of anthrone reagent (2 mg. anthrone per ml. of concentrated H_2SO_4) were added (Dreywood, 1946). The intensity of the resultant coloured solution was measured after 10 minutes in a Coleman colorimeter using a red filter No. 8–215. A blank area of the chromatogram received the same procedure. The amount of sugar present was then determined using a linear calibration curve which had identical slopes for all of the sugars used.[1] A further aliquot of the eluate from the chromatogram was plated and its radioactivity determined. From these data the total activity and specific activity could be calculated.

EXPERIMENTAL RESULTS

The essential data are assembled in a series of tables which form part of this paper, although their full interpretation will extend to subsequent parts of this group of papers.

Growth in terms of fresh weight. These data will be found in Table II. Although significant increase of fresh weight occurred in the tissue cultures not treated with coconut milk, the great contrast between these and the cultures so treated is readily apparent. Under the tissue-culture conditions employed the growth of single explants in a culture tube can be quite accurately duplicated (Caplin and Steward, 1949). It is to be expected, however, that when larger numbers of such explants are placed in the tube somewhat greater variation will obtain. For this reason, therefore, no significance is attached to the differences that are to be seen in the growth of cultures exposed to the different radioactive substances employed. It may be mentioned here that the difference in weight between the tissue not treated with coconut milk and the tissue so treated is not the only criterion of the difference between these two states. It has been shown (Steward, Caplin, and Shantz, 1955; Steward and Shantz, 1956) that the tissue cultured on coconut milk is made up of a large number of small cells, whereas the tissue not so treated contains cells which expand even to a larger size than in the original tissue explant.

Respiration of the tissue. Data as μg. carbon dioxide per 10 explants per hour are shown in Table II. The total carbon, the total radioactivity, and the

[1] The most important aspects of this technique are (i) that the concentrated sulphuric acid must be as dry as possible (at least 96 per cent. H_2SO_4), and (ii) that the anthrone reagent must be added very rapidly to the sugar solution, using a pipette which has had the constricted tip removed, so that mixing is immediately complete and the mixture boils briefly from the heat of mixing. Colour is fully developed after 10 min. and remains stable for several hours. The three sugars here measured gave identical densities per unit of hexose; the calibration curve in the range 5 to 100 μg./ml. was a straight line passing through the origin. Using the Coleman colorimeter a density reading of 0·50 was recorded for a concentration of 50 μg. hexose per ml. It is necessary to remove, by centrifuging, all traces of lint or dust from the solutions to be analysed. The use of filter-aid or Celite when centrifuging or filtering produces erratic results, but other impurities (proteins, soluble nitrogen compounds, salts, &c.) have no effect when present in reasonable amounts.

TABLE II

Growth (mg. fresh wt./10 explants) and respiration (μg. CO_2/hour/10 explants) as affected by time with and without coconut milk (C.M.) in presence of C^{14}-labelled compounds. Time (hours) is reckoned from the addition of the labelled compounds which were added after 7 days of culture when slow (no C.M.) explants weighed 50 mg. and fast (+C.M.) weighed 132·5 mg. At period 5, control explants without C^{14} additions weighed 74·5 mg. (no C.M.), 272 mg. (+C.M.)

	Slow growing (no C.M.)					Fast growing (+C.M.)				
Period	1	2	3	4	5	1	2	3	4	5
Addition Hrs.	0–22	22–40	40–61	61–85	85–109	0–22	22–40	40–61	61–85	85–109
Growth (wts at end of period)										
C^{14}-glucose . .	46·5	62·0	61·5	—	84	138	180	183	—	298
C^{14}-γ-aminobutyric acid . .	57·5	65·0	64·0	—	74	151	157	187	—	244
C^{14}-glutamine .	52·0	59·5	61·0	—	70	168	190	206	—	300
Respiration (average during each period)										
C^{14}-glucose . .	22	24	25	19	18	88	104	94	96	94
C^{14}-γ-aminobutyric acid . .	22	24	26	22	19	89	94	94	86	86
C^{14}-glutamine .	20	20	23	16	17	98	96	98	94	96

specific activity of the respired carbon dioxide are shown in Table III. In the first period the rate of respiration was about 0·73 mg. CO_2/g./hour for the fast-growing cultures in the presence of coconut milk; for the slow-growing cultures in the absence of coconut milk, it was approximately half this rate. These rates may be compared with the known rates of respiration of thin slices of carrot tissue which are of the order of 0·3 mg. CO_2/g. fresh wt./hour for fresh-cut tissue decreasing to 0·05 mg. after 200 hours (Turner, 1940). The different treatments with C^{14}-labelled compounds (Table II) did not affect the respiration rates of the tissue significantly.

For each period an estimate has been made of the percentage of the carbon dioxide which was derived from the radioactive source. This estimate was made as follows. The specific activity of the carbon in the respired carbon dioxide (Table III) was expressed as a percentage of the specific activity of the carbon in the compound furnished; this is the percentage of the respired carbon dioxide which was derived from the radioactive source. The data of Table III show that the two nitrogen compounds contributed appreciably to the respiration, glutamine being more effective than γ-aminobutyric acid. Thirty to 75 per cent. of the respired carbon dioxide came from the C^{14}-glucose; the remainder must have originated from some endogenous source.

The uptake and loss of radioactivity. In Table IV are the data which show the residual radioactivity in the external solution, the radioactivity lost in the form of carbon dioxide and, by difference, the amount which remained in the tissue. The figures for the amount of radioactivity remaining in the tissue probably represent maximum values. They are, however, given as the best indication of the total absorption of the C^{14}-labelled compounds. A balance

TABLE III

Production of CO_2 and $C^{14}O_2$ per 10 carrot explants as affected by time, growth with and without coconut milk (C.M.) and presence of C^{14}-additions. Quantities given are mg. total CO_2—C (C); total counts $\times 10^{-3}$ per minute (cpm); specific activity as cts. $\times 10^{-3}$/min./mg. C. (SpA); per cent. derived from C^{14} addition (%)

			Slow growing (no C.M.)					Fast growing (+C.M.)				
		Period	1	2	3	4	5	1	2	3	4	5
Addition	*Resp.*	$-CO_2$ Hrs.	0–22	22–40	40–61	61–85	85–109	0–22	22–40	40–61	61–85	85–109
C^{14}-glucose	C		0·133	0·119	0·144	0·121	0·115	0·524	0·508	0·544	0·632	0·620
Spec. activity	cpm		1·80	1·82	2·08	3·12	3·68	9·12	9·78	13·12	18·7	19·4
42×10^3 cts./min./mg. C.	SpA		13·6	14·1	14·4	25·8	32·1	17·4	19·3	24·1	29·6	31·2
	%		32	34	35	61	76	41	46	57	70	74
C^{14}-γ-aminobutyric	C		0·133	0·120	0·146	0·131	0·124	0·536	0·460	0·524	0·570	0·570
Spec. activity	cpm		8·23	9·03	12·21	13·9	15·1	25·5	31·6	40·8	57·4	72·0
890×10^3 cts./min./mg. C.	SpA		61·6	75·1	83·4	106	122	47·7	68·7	77·8	101	126
	%		6·94	8·44	9·35	11·9	13·7	5·36	7·71	8·75	11·3	14·1
C^{14}-glutamine	C		0·128	0·098	0·138	0·108	0·110	0·582	0·468	0·576	0·610	0·620
Spec. activity	cpm		21·4	15·9	21·0	24·2	24·8	89·2	77·0	93·0	132	136
890×10^3 cts./min./mg. C.	SpA		171	155	151	224	226	153	163	167	210	219
	%		19·2	17·4	17·0	25·2	25·8	17·2	18·4	18·8	24·3	24·6

TABLE IV

The total radioactivity (thousands of counts per min.) per 10 carrot explants as affected by time, growth with and without coconut milk and in presence of C^{14}*-labelled compounds*

Slow-growing cultures (no C.M.)

		Period no.	1	2	3	5
		Time (hrs.)	22	40	61	109
C^{14} compd. supplied	Total radio activity supplied	Activity in fraction containing C^{14}				
C^{14}-glucose (1·025 mg.)	823	Ext. soln.	697	663	615	632
		Resp. CO_2	1·80	3·62	5·70	12·5
		Tissue	124	156	202	179
C^{14}-γ-aminobutyric (1·935 mg.)	802	Ext. soln.	650	620	600	522
		Resp. CO_2	8·20	17·3	29·5	58·5
		Tissue	144	165	173	221
C^{14}-glutamine (1·970 mg.)	805	Ext. soln.	579	478	406	302
		Resp. CO_2	21·3	37·3	58·3	107·3
		Tissue	205	290	340	395

Fast-growing cultures (+C.M.)

		Period no.	1	2	3	5
		Time (hrs.)	22	40	61	109
C^{14}-compd. supplied	Total radio activity supplied	Activity in fraction containing C^{14}				
C^{14}-glucose (4·10 mg.)	3,308	Ext. soln.	2,450	2,360	2,290	2,230
		Resp. CO_2	9·12	18·9	32·0	70·1
		Tissue	834	912	966	987
C^{14}-γ-aminobutyric (7·74 mg.)	3,210	Ext. soln.	2,360	2,280	2,160	1,930
		Resp. CO_2	25·5	57·1	98·0	227
		Tissue	824	870	954	1,056
C^{14}-glutamine (7·88 mg.)	3,300	Ext. soln.	2,100	1,960	1,740	1,100
		Resp. CO_2	89·2	166	259	527
		Tissue	1,034	1,090	1,220	1,585

sheet showing the recovery of this absorbed radioactivity will not be attempted, however, it may be stated that the various compounds that have been determined do not account for all the activity in the tissue. This is in part due to the many unknown compounds whose activity was not determined. Various tests have shown that appreciable losses did not occur during the extraction and chromatographic procedures.

The nitrogeneous constituents of the tissue. The free and combined amino-acids of all the samples are expressed in terms of μg./10 explants in Tables V to XI.

Table V shows the relative composition of the tissue, with and without coconut milk, at the outset of the main experimental treatments. In other work (Steward and Thompson, 1954; Steward, Thompson, and Pollard, 1957) certain contrasts between the nitrogenous composition of the rapidly growing tissue, in the presence of coconut milk, and the slow-growing tissue, in its absence, have been recognized. Notably the protein nitrogen fraction of the actively growing cultures contains hydroxyproline as a rather conspicuous amino-acid. This is shown in Table V. There is also a marked tendency for

the compounds related to glutamic acid, viz. glutamic acid, glutamine and γ-aminobutyric acid, taken together, to predominate in the faster-growing tissue, over the compounds related to aspartic acid, viz. aspartic acid and asparagine. In the original storage tissue, or in the tissue exposed to basal medium without the coconut milk, this is not so. However, the main purpose of Table V is not so much to present a detailed contrast between these two types of cultured tissue as to furnish the baseline from which the further changes induced by the experimental treatments were determined.

Table VI shows the content, per 10 tissue explants, of the free and combined nitrogen compounds, at the various time periods for the tissue treated with C^{14}-labelled glucose. Similarly, Table VII gives the data for the tissue treated with C^{14}-labelled γ-aminobutyric acid, and Table VIII for the tissue treated with C^{14}-labelled glutamine. Interpretations of these data are to follow.

The specific activity of carbon in various compounds. The data from the treatments with the labelled nitrogen compounds are shown in Tables IX to XII for periods 1 to 5 and Table V for the condition at period 0. The corresponding data for the treatment with C^{14}-labelled glucose are in Table XV. Specific activities are expressed as a percentage of the activity of the carbon in the labelled compound as supplied to the tissue. The specific activity data provide more information on the sequence of the formation of the compounds in question than is available merely from the measurement of their total radioactivity. The assumption is often made that the more nearly the specific activity of the carbon in a metabolite approaches the specific activity of the carbon in the source, the closer this substance must be to the source in the sequence of reactions involved in its formation. The way in which this idea worked out in the present problem will be scrutinized in Part II.

Only a limited range of carbon compounds became strongly radioactive when the tissue was furnished with C^{14}-labelled glucose (Table XV). This may have been due in part to the lower specific activity of the carbon source.

Tables IX to XII present the data for the amounts of carbon and the activity present in free and combined amino-acids in the tissue treated with C^{14}-labelled nitrogen compounds at the different periods involved in the experiment. From the appropriate chromatographic analysis, the amount of total carbon (in μg.) represented by the content of that compound in 10 tissue explants was calculated (C). The radioactivity corresponding to this amount of carbon is given in thousands of counts per minute (A). This is designated the total activity of the carbon in the compound in question. The 'carbon equivalent of the activity', designated 'C ≡ Activity', shortened to CA, represents the amount of carbon in μg., of the same specific activity as the source, which would have produced the activity measured. In other words, this represents μg. carbon now present in the compound analysed, which was derived from the C^{14}-compound supplied. The term 'per cent. specific activity', designated as '% S.A.' in the tables, is a measure of the specific activity of the carbon in the compound in question as a percentage of that in the compound supplied.

Tables XIII and XIV derive from data contained in the Tables IX to XII; these require the following explanation.

In a metabolic system, the situation will frequently arise that a given compound is subject during a given time period to increase from one source and decrease from another, that is, there may be both entry and withdrawal from what might be described as the metabolic pool. In such a situation it is not enough merely to know the specific activity of the carbon in the substance contained in that metabolic pool at any one time. The additional information that is required is the specific activity of the carbon which is in transit to and from the metabolite in question. In order to obtain this, the following data are necessary.

(i) The change in a given period in the total carbon contained in the metabolite in question.

(ii) The change in the same period in the activity contained in the metabolite in question.

From this information the specific activity of the carbon which is in transit may be calculated. This is achieved by dividing (ii) by (i) above, i.e. by dividing the change in the activity by the change in the carbon.

For purposes of convenience, however, the calculation mentioned above has been carried out in a slightly different way, by using units of carbon instead of units of specific activity. That is, the carbon equivalent of the activity has been substituted for the absolute measurement of activity in terms of counts per minute per unit of carbon. This is done because the figures in relative units of carbon are more useful for comparative purposes than are the data in terms of absolute specific activity. Carrying out the calculation in this manner involves the use of the following terms which are conveniently represented by the symbols shown.

Δ total C (written ΔC), represents the change (μg.) in total carbon in the substance in question from one period to the next.

$\Delta C \equiv$ activity (written ΔCA), represents the change (μg.) from one period to the next in the *carbon equivalent of the activity* present in the form of the substance in question.

$\Delta C \equiv$ activity $\times 100/\Delta$ total C (written $100\Delta CA/\Delta C$), represents the specific activity of the carbon *appearing in, or disappearing from,* the pool of the substance in question from one period to the next. This activity is expressed as a percentage of the specific activity of the carbon in the C^{14}-compound supplied.

The data which result from this form of analysis are contained in Tables XIII (soluble compounds) and XIV (insoluble compounds). It is apparent that some figures for the specific activity of the carbon which is in transit, may be greater than 100, that is, the *carbon apparently had specific activity greater than that supplied.* In other parts of the tables the data, if calculated, could give figures for the specific activity of carbon in transit which approach infinity, or, in other situations, have a negative value. It is the significance of these apparently anomalous results, as well as the general interpretation of this type of data, that now requires to be analysed.

TABLE V

The free and combined amino-acids (μg./10 explants) of the carrot tissue to which the C^{14}-labelled compounds were to be applied (i.e. after 7 days of culture treatment)

		Slow-growing cultures (no C.M.)		Fast-growing cultures (+C.M.)	
		Free	Combined	Free	Combined
2	Aspartic	12·0 (4·3)	25·0 (9·0)	11·2 (4·0)	80·2 (28·9)
3	Glutamic	19·2 (7·8)	39·6 (16·2)	63·0 (25·7)	116·0 (47·3)
4	Serine	3·1 (1·1)	18·0 (6·1)	16·5 (5·7)	52·9 (18·1)
5	Glycine	— —	16·8 (5·4)	— —	57·3 (18·3)
6	Asparagine	17·8 (6·5)	— —	34·4 (12·5)	— —
7	Threonine	— —	22·5 (9·0)	— —	53·9 (21·7)
8	Alanine	6·5 (2·6)	19·7 (7·9)	23·5 (9·5)	63·6 (25·6)
9	Glutamine	14·7 (6·0)	— —	13·5 (5·5)	— —
11	Histidine	— —	— —	— —	— —
12	Lysine	— —	11·7 (5·8)	— —	56·9 (28·1)
13	Arginine	— —	9·0 (3·7)	— —	35·3 (14·6)
15	Proline	— —	38·7 (20·2)	— —	76·3 (39·8)
16	Valine	5·2 (2·7)	20·2 (10·4)	13·0 (6·7)	58·6 (30·0)
18	Leucine	— —	34·5 (19·0)	— —	116·0 (63·8)
19	Phenylalanine	— —	— —	— —	90·9 (59·5)
21	Tyrosine	— —	— —	— —	— —
23	γ-aminobutyric	3·5 (1·6)	— —	14·5 (6·8)	— —
24	Hydroxyproline	— —	trace —	— —	37·6 (17·2)

Numbers in parentheses represent the carbon content in μgms. of the amino-acids (free or combined) as determined.

TABLE VI

The total quantity (μg.) of each combined amino-acid (P) or free amino-acid (F) per 10 carrot explants as affected by time, growth with and without coconut milk and in presence of C^{14}-labelled glucose

Amino-acid		Period no.	Slow-growing cultures (no C.M.)				Fast-growing cultures (+C.M.)			
			1	2	3	5	1	2	3	5
No.	*Identity*									
2	Aspartic	P	32·0	35·0	38·0	50·0	80·0	112	144	185
		F	trace	6·4	3·57	7·5	—	trace	trace	trace
3	Glutamic	P	38·5	40·7	49·5	56·5	110·0	147	176	211
		F	20·9	20·9	13·4	22·2	59·0	70·6	73·5	97·4
4	Serine	P	18·7	18·7	27·9	30·6	64·0	54·5	70·4	83·1
		F	trace	—	trace	—	11·5	13·0	13·5	17·3
5	Glycine	P	21·8	22·2	23·9	31·3	47·0	93·3	109·0	120
6	Asparagine	F	trace	—	trace	—	—	—	trace	trace
7	Threonine	P	23·1	25·0	31·1	32·8	53·1	77·7	114·0	106
8	Alanine	P	21·5	21·2	25·9	31·8	62·8	84·3	95·8	109
		F	8·85	2·50	6·67	4·37	24·0	(24·2)	40·8	46
9	Glutamine	F	22·0	10·20	11·50	11·9	15·0	(18)	28·9	(34)
12	Lysine	P	14·0	17·1	16·2	12·9	36·2	83·7	76·9	90·6
13	Arginine	P	1·65	10·9	14·7	13·1	26·2	60·0	62·1	70·0
15	Proline	P	43·7	5·0	47·9	54·3	127·0	127·0	214·0	203·0
		F	25·6	—	—	—	—	—	—	—
16	Valine	P	25·7	24·5	30·8	34·2	53·0	76·9	108·0	97·2
		F	—	trace	trace	3·35	4·4	9·35	16·2	27·5
18	Leucine	P	39·7	38·7	50·3	58·2	91·0	155·0	59·4	185·0
		F	—	—	—	—	—	—	—	—
19	Phenylalanine	P	—	—	—	trace	—	140	—	—
21	Tyrosine	P	some	some	some	some	some	some	some	some
23	γ-aminobutyric	F	5·92	trace	9·37	5·62	9·4	13·1	13·7	20·8
24	Hydroxyproline	P	18·7	34·7	7·3	52·1	some	77	107·5	25·9
26	Pipecolic	F	trace	—	—	—	—	—	—	—

Histidine detected but not determined. Figures in parentheses are estimates because separations were not so good as desired.

TABLE VII

The total quantity (μg.) of each combined amino-acid (P) or free amino-acid (F) per 10 carrot explants as affected by time, growth with and without coconut milk and in presence of C^{14}-labelled γ-aminobutyric acid

Amino-acid		Period no.	Slow-growing cultures (no C.M.)				Fast-growing cultures (+C.M.)			
			1	2	3	5	1	2	3	5
No.	*Identity*									
2	Aspartic	P	38·1	42·3	33·5	46·1	97·5	128	173	181
		F	trace	trace	4·77	7·03	trace	trace	9·55	6·67
3	Glutamic	P	45·0	48·9	44·4	52·3	117	147	221	209
		F	22·2	30·3	27·5	26·5	58·0	72·5	103	154
4	Serine	P	24·1	30·9	22·2	28·7	9·4	70·8	96·9	78·9
		F	2·96	4·45	6·50	5·28	15·5	17·5	26·6	39·3
5	Glycine	P	23·4	25·0	19·7	34·1	65·6	76·9	117	136
6	Asparagine	F	trace	trace	37·0	32·6	trace	111	109	68·8
7	Threonine	P	27·4	31·3	27·4	29·7	62·3	75·2	122	110
		F	—	—	—	—	—	—	—	15
8	Alanine	P	25·7	29·2	23·4	33·6	61·5	79·1	111	114
		F	15·32	21·41	21·1	34·3	87·0	142	305	542
9	Glutamine	F	76·5	119	140	321	232	318	697	904
12	Lysine	P	15·8	19·5	14·7	25·5	52·7	71·9	100	121
13	Arginine	P	11·2	16·3	3·75	19·2	39·8	34·2	74·4	75·6
15	Proline	P	70·3	70·7	52·2	36·8	50·4	86·0	201	155
		F	38·9	40·31	50·6	54·6	trace	—	67·2	96·7
16	Valine	P	27·6	29·2	27·5	31·0	62·7	70·2	117	93·6
		F	4·22	10·59	15·6	16·1	32·9	36·6	53·1	68·8
18	Leucine(s)	P	49·4	52·1	43·8	59·9	118	122	216	196
		F	—	—	—	5·23	—	—	20·4	25·0
19	Phenylalanine	P	some	45·3	—	60·1	—	—	trace	trace
21	Tyrosine	P	some	some	some	some	—	some	some	some
23	γ-aminobutyric	F	39·15	40·31	30·1	30·4	171	181	292	260
24	Hydroxyproline	P	46·5	4·17	2·5	59·9	33·1	48·1	50·1	50·0

Histidine detected but not determined.

Table VIII

The total quantity (μg.) of each combined amino-acid (P) or free amino-acid (F) per 10 carrot explants affected by time, growth with and without coconut milk and in presence of C^{14}-labelled glutamine

Amino-acid*		Period no.	Slow-growing cultures (no C.M.)				Fast-growing cultures (+C.M.)			
			1	2	3	5	1	2	3	5
No.	*Identity*									
2	Aspartic	P	27·7	31·2	46·1	54·7	some	110	164	244
		F	trace	7·0	13·9	9·75	18·5	12·3	17·5	15·0
3	Glutamic	P	30·5	35·5	50·9	57·2	some	141	208	221
		F	27·5	27·7	34·2	40·0	109	119	137	184
4	Serine	P	20·2	19·8	29·4	24·8	—	50·5	94·6	80·8
		F	4·25	6·75	6·95	14·7	16·0	20·2	38·0	46·4
5	Glycine	P	18·3	15·0	23·4	32·6	—	95·0	119	171
6	Asparagine	F	25·8	36·4	12·8	30·3	61·6	109	141	202
7	Threonine	P	19·1	22·2	33·7	31·9	—	72·9	127	117
8	Alanine	P	17·2	18·1	28·7	28·8	—	84·0	103	128
		F	13·2	19·0	25·7	—	135	311	459	642
9	Glutamine	F	115	283	405	757	647	832	1,370	2,228
12	Lysine	P	4·67	5·72	13·2	15·8	—	87·2	106	150
13	Arginine	P	2·50	3·75	17·3	8·5	some	52·1	100	80·0
15	Proline	P	0·95	43·7	42·2	60·4	—	101	281	195
		F	25·2	trace	43·3	41·5	—	trace	75·8	trace
16	Valine	P	19·7	20·0	33·2	27·8	—	71·6	115	106
		F	8·75	14·7	16·1	19·6	32·9	40·9	56	82
18	Leucine(s)	P	29·4	28·9	52·1	48·7	—	133	202	213
		F	—	—	—	—	12·1	trace	22·5	26·1
19	Phenylalanine	P	—	—	—	—	—	57·5	—	174
21	Tyrosine	P	—	—	some	some	—	some	some	some
23	γ-aminobutyric	F	5·62	5·9	9·62	4·7	19·6	25·6	37·5	57·5
24	Hydroxyproline	P	26·9	some	34·4	29·8	some	41·9	some	143

* Though detected on almost all chromatograms, histidine was not determined. Pipecolic acid trace in period 1.

Table IX

The carbon in the soluble compounds of the tissue treated with C^{14}*-γ-aminobutyric acid [C = total carbon (μg.); A = total activity (cts per min.* $\times 10^{-3}$*); CA = carbon equiv. (μg.) of the activity; % SA = % specific activity] as affected by time and conditions of growth: all data per 10 carrot explants*

		Slow-growing cultures (no C.M.)				Fast-growing cultures (+C.M.)			
	Period no.	1	2	3	5	1	2	3	5
	Time (hrs.)	22	40	61	109	22	40	61	109
Compounds:									
γ-aminobutyric	C	18·2	18·8	14·0	25·4	79·7	84·6	136	121
	A	15·1	13·1	13·5	16·2	81·8	90·7	102	94·3
	CA	17·0	14·7	15·2	18·2	91·9	102	115	106
	% SA	93	78	120	72	115	115	84	87
Glutamic acid	C	9·1	12·4	11·2	19·4	23·7	29·6	42·2	62·8
	A	1·95	3·4	3·0	4·2	6·1	8·6	11·6	14·5
	CA	2·2	3·8	3·4	4·7	6·8	9·7	13·0	16·3
	% SA	24	31	31	24	29	33	31	26
Glutamine	C	31·4	49·1	57·6	237·3	95·3	131	287	371
	A	9·0	7·6	10·5	28·0	12·1	20·0	28·4	36·0
	CA	10·1	8·5	11·8	31·4	13·6	22·5	31·9	40·5
	% SA	32	17	20	13	14	17	11	11
Proline	C	—	21·0	26·4	51·2	—	—	—	trace
	A	insig.	0·28	0·75	3·0	insig.	insig.	insig.	1·9
	CA	—	0·30	0·85	3·4	—	—	—	2·1
	% SA	—	1·5	3·0	6·5	—	—	—	—
Asparagine	C	trace	trace	13·5	21·3	trace	40·3	39·5	25·0
	A	0·2	0·45	1·05	3·2	0·4	1·9	4·0	6·1
	CA	0·22	0·50	1·20	3·6	0·45	2·1	4·5	6·8
	% SA	—	—	9·0	17·0	—	5·0	11·0	27·0
Threonine	C	—	—	—	—	—	—	—	6·0
	A	insig.	insig.	insig.	insig.	insig.	insig.	0·2	0·6
	CA	—	—	—	—	—	—	0·2	0·7
	% SA	—	—	—	—	—	—	—	12·0
Glucose and sucrose	% SA	—	—	—	3·4	—	—	—	1·1
Fructose	% SA	—	—	—	3·4	—	—	—	insig.

Alanine did not become significantly radioactive in tissue treated with γ-aminobutyric acid.

TABLE X

Carbon in the combined amino-acids of the tissue treated with C^{14}*-γ-aminobutyric acid. Conventions as for Table IX*

		Slow growing (no C.M.)				Fast growing (+C.M.)			
Period no.		1	2	3	5	1	2	3	5
Time (hrs.)		22	40	61	109	22	40	61	109
Compounds:									
Glutamic acid	C	18·4	20·0	18·1	21·3	47·7	60·2	90·2	85·4
	A	0·45	0·78	0·80	1·53	1·0	1·4	5·0	6·5
	CA	0·51	0·88	0·90	1·72	1·12	1·57	5·6	7·3
	% SA	2·7	4·4	5·0	8·1	2·3	2·6	6·2	8·5
Proline	C	36·7	36·9	27·2	19·2	26·3	44·9	105	80·9
	A	0·15	0·28	0·45	0·77	0·37	0·65	1·28	2·7
	CA	0·17	0·31	0·51	0·87	0·42	0·73	1·44	3·0
	% SA	0·46	0·84	1·9	4·5	1·6	1·6	1·4	3·7
Hydroxyproline	C	21·3	1·9	1·1	27·4	15·2	22·0	23·0	22·9
	A	0·18	0·44	0·5	1·17	0·3	0·4	0·6	2·1
	CA	0·20	0·49	0·56	1·31	0·34	0·45	0·67	2·4
	% SA	0·94	26	51	4·8	2·2	2·0	2·8	10·5
Aspartic acid	C	13·8	15·3	12·1	16·6	35·2	46·4	62·5	65·5
	A	0·5	N.D.	0·75	1·33	0·9	N.D.	N.D.	6·0
	CA	0·56	—	0·84	1·50	1·0	—	—	6·7
	% SA	4·1	—	6·9	9·0	2·9	—	—	10·2
Threonine	C	11·0	12·6	11·0	12·0	25·1	30·3	49·4	44·5
	A	0·10	0·11	0·20	0·33	N.D.	0·3	0·8	2·0
	CA	0·11	0·12	0·22	0·37	—	0·34	0·9	2·2
	% SA	1·0	0·95	2·0	3·1	—	1·1	1·8	4·9

TABLE XI

The carbon in the soluble compounds of the tissue treated with C^{14}-glutamine. Conventions as in Table IX

		Slow growing (no C.M.)				Fast growing (+C.M.)			
Period no.		1	2	3	5	1	2	3	5
Time (hrs.)		22	40	61	109	22	40	61	109
Compounds:									
γ-aminobutyric	C	2·6	2·8	4·5	3·0	9·1	11·9	17·5	26·8
	A	0·45	0·40	0·62	0·60	2·4	2·1	4·2	6·0
	CA	0·50	0·45	0·70	0·67	3·0	2·4	4·7	6·7
	% SA	19	14	15	31	29	20	27	25
Glutamic acid	C	11·2	11·3	13·9	16·3	44·7	48·5	55·9	75·1
	A	6·2	4·5	8·6	8·5	39·4	36·5	40·1	54·3
	CA	7·0	5·1	9·7	9·5	44·3	41·0	45·0	61·0
	% SA	62	45	69	58	99	84	81	81
Glutamine	C	47·4	116·4	166	311	266	342	563	916
	A	35·5	62·0	102	120	155	177	230	319
	CA	39·9	69·7	114	135	175	198	258	358
	% SA	84	60	69	43	66	58	46	39
Proline	C	13·1	trace	22·6	21·7	—	trace	39·6	trace
	A	0·4	1·2	2·3	3·8	0·9	1·1	2·8	4·7
	CA	0·45	1·4	2·6	4·3	1·0	1·2	3·1	5·3
	% SA	8	—	13	19	—	—	8·0	—
Asparagine	C	9·4	13·2	4·7	11·7	22·4	39·8	51·2	73·7
	A	0·7	1·2	2·8	4·6	insig.	5·1	9·1	17·7
	CA	0·8	1·3	3·1	5·2	—	5·7	10·1	19·9
	% SA	8	10	72	47·0	—	14	20	27
Threonine	C	—	trace	trace	—	—	trace	—	—
	A	0·8	0·8	1·2	1·2	0·6	1·9	1·2	1·6
	CA	0·9	0·9	1·3	1·3	0·7	1·1	1·3	1·8
	% SA	—	—	—	—	—	—	—	—
Alanine	C	5·3	7·7	—	—	—	—	—	—
	A	0·7	1·0	•	•	—	—	—	—
	CA	0·8	1·1	—	—	—	—	—	—
	% SA	15	14	—	—	—	—	—	—
Glucose and sucrose	% SA	—	—	—	5·6	—	—	—	1·4
Fructose	% SA	—	—	—	6·6	—	—	—	insig.

• In these cultures the activity of glutamine superimposed on alanine which could not therefore be determined. Hydroxyproline was not detected by ninhydrin or by radioactivity in the tissue receiving C^{14} glutamine.

TABLE XII

Carbon in the combined amino-acids of the tissue treated with C^{14}-glutamine. Conventions as in Table IX

		Slow growing (no C.M.)				Fast growing (+C.M.)			
Period no.		1	2	3	5	1	2	3	5
Time (hrs.)		22	40	61	109	22	40	61	109
Compounds:									
Glutamic acid	C	12·4	14·5	21·9	23·3	N.D.	57·7	84·9	90·2
	A	0·75	1·0	3·8	N.D.	N.D.	11·3	22·6	22·5
	CA	0·84	1·1	4·3	—	—	12·7	25·4	25·3
	% SA	6·8	7·7	19·6	—	—	22	30	28
Proline	C	0·47	22·8	22·0	31·5	N.D.	52·8	147	102
	A	0·55	0·5	1·4	2·25	N.D.	3·8	6·0	12·9
	CA	0·62	0·56	1·57	2·53	—	4·3	6·7	14·5
	% SA	132	2·5	7·1	8·0	—	8·1	4·6	14
Hydroxyproline	C	12·3	0·55	11·2	13·6	N.D.	19·2	N.D.	65·5
	A	0·60	0·7	1·5	3·2	1·20	2·6	4·8	12·4
	CA	0·67	0·79	1·69	3·6	1·35	2·9	5·4	13·9
	% SA	5·4	113	15·1	26	—	15	—	21
Aspartic acid	C	10·0	11·3	16·6	19·7	N.D.	39·9	59·2	88·1
	A	0·65	0·85	N.D.	N.D.	N.D.	4·1	15·2	17·4
	CA	0·73	0·96	—	—	—	4·6	17·1	19·5
	% SA	7·3	8·5	—	—	—	11	29	22
Threonine	C	—	8·9	13·6	12·9	N.D.	29·4	51·4	47·0
	A	—	0·1	0·28	0·6	N.D.	1·2	2·4	2·7
	CA	—	0·11	0·31	0·67	—	1·35	2·7	3·0
	% SA	—	1·2	2·3	5·2	—	4·6	5·2	6·4
Alanine	C	—	—	—	—	N.D.	33·9	41·7	51·7
	A	—	—	—	—	N.D.	0·3	N.D.	1·2
	CA	—	—	—	—	—	0·34	—	1·3
	% SA	—	—	—	—	—	1·0	—	2·5

Table XIII

The movement of carbon, total and radioactive, as μg. per 10 carrot explants into free amino-acids as affected by time, conditions of growth, and the C^{14}-labelled substance supplied

ΔC represents the change (μg.) in total carbon in the substance in question from one period to the next.

ΔCA represents the change (μg.) from one period to the next in the carbon equivalent of the activity present in the form of the substance in question.

100ΔCA/ΔC represents the specific activity of the carbon appearing in, or disappearing from, the pool of the substance in question from one period to the next, expressed as percentage of the specific activity of the carbon in the compound supplied.

		Slow-growing cultures (no C.M.)					Fast-growing cultures (+C.M.)				
	Period no.	1	2	3	4–5	1–5	1	2	3	4–5	1–5
	Duration (hrs.)	0–22	22–40	40–61	61–109	0–109	0–22	22–40	40–61	61–109	0–109
Source of activity = C^{14} γ-aminobutyric acid											
Compound:											
γ-aminobutyric	ΔC	16·8	0·5	−4·7	11·4	23·8	72·8	4·9	51·7	−15·4	114
	ΔCA	17·0	−2·3	0·5	3·0	18·2	91·9	10	13·1	−9·0	106
	100ΔCA/ΔC	101	—	—	27	76	126	204	25	58·5	93
Glutamic acid	ΔC	1·2	3·3	−1·1	8·2	11·6	−2·0	5·9	12·6	20·6	37·1
	ΔCA	2·2	1·6	−0·4	1·3	4·7	6·8	2·9	3·3	3·3	16·3
	100ΔCA/ΔC	182	49	36	16	41	—	49	26	16	44
Glutamine	ΔC	25·4	17·6	8·6	179	231	89·8	35·5	156	84·8	366
	ΔCA	10·1	−1·6	3·3	19·6	31·4	13·6	8·9	9·4	8·6	40·5
	100ΔCA/ΔC	40	—	39	11	14	15	25	6	10	11
Proline	ΔC	—	0·7[a]	5·4	24·8	30·9[b]	—	—	—	—	—
	ΔCA	—	0·3	0·5	2·5	3·4	—	—	—	—	—
	100ΔCA/ΔC	—	43	10	10	11	—	—	—	—	—
Source of activity = C^{14} glutamine											
Compound:											
γ-aminobutyric	ΔC	1·0	0·2	1·7	−2·3	0·56	2·3	2·8	5·6	9·3	20·1
	ΔCA	0·5	−0·1	0·3	0·0	0·67	2·7	−0·3	2·3	2·0	6·7
	100ΔCA/ΔC	51·5	—	18	0·0	120	117	—	41	22	33
Glutamic acid	ΔC	3·3	0·1	2·6	2·3	8·4	19	3·8	7·4	19·2	49·4
	ΔCA	7·0	−1·9	4·6	−0·1	9·5	44·3	−3·3	4·0	16·0	61·0
	100ΔCA/ΔC	208	—	173	—	112	233	—	54	83	123
Glutamine	ΔC	41·3	69	50·1	145	305	261	76	221	352	910
	ΔCA	39·9	29·8	44·6	20·5	135	175	23·8	60·0	99·8	358
	100ΔCA/ΔC	97	43	89	14	44	67	31	27	28	39
Asparagine	ΔC	2·9	3·9	−8·6	6·4	4·5	9·9	17·4	11·4	22·5	61·2
	ΔCA	0·8	0·6	1·8	2·1	5·2	0·0	5·7	4·4	9·8	19·9
	100ΔCA/ΔC	27	14	—	33	115	0·0	33	39	44	32

[a] Period 1–2, not 2.

[b] Period 2–5 not 1–5.

TABLE XIV

The movement of carbon, total and radioactive, as μg. per 10 carrot explants, into combined amino-acids, as affected by time, conditions of growth, and the C^{14}-labelled substance supplied. Conventions as in Table XIII

		Slow-growing cultures (no C.M.)					Fast-growing cultures (+C.M.)				
Period no.		1	2	3	4–5	1–5	1	2	3	4–5	1–5
Duration (hrs.)		0–22	22–40	40–61	61–109	0–109	0–22	22–40	40–61	61–109	0–109
Source of activity = C^{14}-γ-aminobutyric acid											
Compound:											
Glutamic acid	ΔC	2·3	1·6	−1·9	3·2	5·2	0·4	12·5	30·0	−4·8	38·1
	ΔCA	0·51	0·37	0·02	0·82	1·72	1·1	0·45	4·0	1·7	7·3
	100ΔCA/ΔC	22	23	—	26	33	280	3·6	13	—	19
Proline	ΔC	16·5	0·2	−9·7	−8	−1·0	−13·5	23·8	60·1	−24·1	41·1
	ΔCA	0·17	0·14	0·2	0·36	0·87	0·42	0·31	0·71	1·6	3·0
	100ΔCA/ΔC	1	70	—	—	—	—	1·3	1·2	—	7
Hydroxyproline	ΔC	—	—	—	6·1[b]	6·1	−2·0	6·8	1·0	−0·1	13·4
	ΔCA	0·20	0·29	0·07	1·11	1·11	0·34	0·11	0·22	1·7	2·4
	100ΔCA/ΔC	—	—	—	18	18	—	1·6	22·0	—	18
Aspartic acid	ΔC	4·2	—	−1·7[a]	4·5	7·6	6·2	—	—	30·3[a]	36·5
	ΔCA	0·56	N.D.	0·28	0·66	1·5	1·01	N.D.	N.D.	5·7	6·7
	100ΔCA/ΔC	13	—	—	15	20	16	—	—	19	18
Threonine	ΔC	2·0	1·6	−1·6	1·0	3·0	—	5·2	19·1	−4·9	22·8
	ΔCA	0·11	0·01	0·1	0·15	0·37	—	0·34	0·56	1·3	2·2
	100ΔCA/ΔC	5	0·6	—	15	12	—	6·5	2·9	—	10

Source of activity = C^{14} glutamine							Period no.	1–2	3	4–5	0–5
							Duration (hrs.)	0–40	40–61	61–109	0–109
Glutamic acid	ΔC	−3·7	2·1	7·4	—	5·8[a]		10·4	27·2	5·3	42·9
	ΔCA	0·84	0·28	3·2	N.D.	4·3		12·7	12·7	−0·1	25·3
	100ΔCA/ΔC	—	13	43	—	74		122	47	—	59
Proline	ΔC	—	2·6[d]	−0·8	9·5	11·3		20·7	94	−45·0	62·0
	ΔCA	0·62	0·56	1·0	1·0	2·53		4·3	2·4	7·8	14·5
	100ΔCA/ΔC	—	22	—	10	22		21	2·5	—	23
Hydroxyproline	ΔC	—	—	—	2·4	1·3		2·0	—	46·5[f]	48·3
	ΔCA	0·67	0·12	0·9	2·3	2·9		2·9	2·5	11·0	13·9
	100ΔCA/ΔC	—	—	—	96	223		145	—	24	29
Aspartic acid	ΔC	1·0	1·3	5·3	—	2·3		10·9	19·3	28·9	59·1
	ΔCA	0·73	0·23	N.D.	N.D.	0·96		4·6	12·5	2·4	19·5
	100ΔCA/ΔC	73	18	—	—	42		42	65	8	33
Threonine	ΔC	—	1·2[d]	4·7	−0·7	—		7·7	22·0	−4·4	7·2
	ΔCA	—	0·11	0·2	0·36	—		1·35	1·4	0·3	3·0
	100ΔCA/ΔC	—	9	4·2	—	—		18	6	—	42
Alanine	ΔC	—	—	—	—	—		8·2	7·8	17·8[f]	26·0
	ΔCA	—	—	—	—	—		3·4	N.D.	1·0	1·3
	100ΔCA/ΔC	—	—	—	—	—		4	—	6	5

[a] Periods 2–3, not 3.
[b] Periods 1–5, not 4–5.
[c] Periods 2–5, not 4–5.
[d] Periods 1–2, not 2.
N.D. = Not determined.
[e] Periods 1–4, not 1–5
[f] Periods 3–5, not 4–5.

TABLE XV

Specific activity of carbon, as per cent. of that supplied, in the compounds receiving carbon from C^{14}*-glucose*

	Slow-growing cultures (no C.M.)				Fast-growing cultures (+C.M.)			
Period no.	1	2	3	5	1	2	3	5
Compound:								
CO_2	32	34	35	76	41	46	57	74
Sugars:								
Glucose and sucrose	N.D.	29	33	48	38	55	71	N.D.
Fructose	N.D.	26	29	48	17	40	60	71
Free amino-acids:								
Glutamic acid	—	—	59	86	—	—	95	102
Alanine	—	—	—	—	—	—	40	100
Combined amino-acids:								
Aspartic acid	—	—	—	—	—	—	—	43
Glutamic acid	—	—	24	18	—	—	—	46
Alanine	—	—	—	—	—	—	—	50
Threonine	—	—	—	—	—	—	—	26

N.D. = Not determined. — = Insufficient activity for measurement.

II. THE INTERPRETATION OF SPECIFIC ACTIVITY DATA IN TRACER EXPERIMENTS

WITH the widespread use of radioactivity in tracer experiments the concept of specific activity has become familiar and is usually applied to a metabolic system as follows.

It is generally assumed that in a metabolic sequence, the succeeding members must have lower specific activities than the compound from which they were derived. Furthermore, the sequence of their specific activities is used as a criterion of the order of their formation. This criterion applies only to a homogeneous system in which each reactant exists in, and forms part of, a single freely interchangeable metabolic pool. This criterion, however, does not apply to any situation in which any member of the reaction sequence also exists in a parallel, but not freely interchangeable, metabolic pool which, on the analysis of the entire cell, mingles with the pool in which the reaction sequence occurs. The position becomes even further complicated when the same metabolites occur in different cellular inclusions and participate in quite different but concurrent metabolic reactions:

Some representative examples are as follows:

Take the situation in which compound *A*, of specific activity 100, is supplied to the cell, and there is a pool of *A* already existing which does not mingle with the *A* supplied. The compound *A* as supplied is then converted to the substance *B*, resulting in a distinct pool of *B* which has specific activity 100 (since *B* did not exist previously in the cell). After this conversion the cell is analysed and the specific activity of *A* and *B* are determined, *B* clearly producing a value of 100. However, due to the dilution of the radioactive substance *A*, by the inactive pool of *A* already present which mingled with it

during the extraction process, the specific activity of *A* in the cell as determined is considerably less than 100. In these circumstances it appears that the product of the reaction, *B*, has a higher specific activity than the molecule, *A*, from which it was derived in the cell. This very simple example clearly shows that one cannot always use the specific activity of the compounds *as extracted from the cell* to indicate the sequence of the reactions in which they participate. Moreover, the position becomes further complicated if what has here been described as the inactive cellular pool of the substance *A* now takes part in quite different metabolic reactions than those which lead to the substance *B*. Far from being an unusual situation, that portrayed above should, in fact, be expected, due to the known presence of discrete inclusions in cells, each capable of their own metabolism.

How then may such 'compartments' in the cell with their parallel reactions be detected? This may be accomplished using such data as those in Tables XIII and XIV. Thus one may use the data of specific activity of carbon in transit, to or from metabolites, to detect heterogeneity in the cell, both with respect to the geographical location of the metabolites and with respect to the reactions in which they participate. The concept that is useful in doing this is *the apparent specific activity of the carbon entering or leaving the metabolic pool of a given compound during an interval of time, relative to the specific activity in the source supplied*, i.e. $\Delta C \equiv$ activity $\times 100/\Delta$ total C (written $100\Delta CA/\Delta C$). The utility of this concept may be illustrated by the following simple hypothetical situation:

Let there be two independent cellular inclusions or compartments, here numbered (i) and (ii).

Substance *A* exists in both compartments.

The quantity in each compartment at the beginning of a tracer experiment is set at 50 units of C^{12} in the form of compound *A* in (i), 50 units of C^{14} in the form of *A* in (ii).

At the end of the period of observation, during which C^{14} from a source with a specific activity of 100, enters into compound *A*, the amounts in each compartment were: 30 units of C^{12} in the form of *A* in compartment (i); 80 units of C^{14} in the form of Compound *A* in compartment (ii); and no other radioactive compounds were present in the cell or the medium.

Therefore, during the period:

The change in the total radioactivity in the system was $\equiv$ 30 units of C^{14} each with a specific activity of 100.

The net change in the total carbon in the form of compound *A* in the system was 10 units of C. Therefore, the apparent specific activity of the carbon that enters compound *A in the cell* during the period, measured as a multiple of the specific activity of the source supplied, equals 3 (i.e. the change in total radioactivity expressed in equivalent units of carbon in the compound *A* divided by the actual units of carbon by which this change was brought about).

Thus the apparent specific activity of the carbon which entered the metabolic pool of compound *A* during the period equals 300 measured on a scale on which the C^{14} in the compound supplied had specific activity 100.

It is important now to notice the following: The apparent anomaly that the carbon which enters into the composition of compound *A*, during the period of observation, had specific activity far greater than the carbon in the source from which it was clearly derived, is sufficient indication that compound *A* exists in more than one phase, or compartment, and that the C^{14} is not equally concerned with the different events that affect this compound *A* during the period in question. Thus an anomalous value for the 'specific activity of the carbon which enters or leaves the metabolic pool of a given metabolite in a given period', i.e. $100\Delta CA/\Delta C$, may yield evidence of geographical separation of that compound into different compartments in the cell. It may also indicate that the compound in question may, in its formation and use, be subjected to quite different metabolic situations in these compartments. If the metabolizing system is a heterogeneous tissue system, the compartments may, of course, be at the cellular level. However, if the metabolizing system consists of a statistically homogeneous system of cells, as in the present experiment, the compartments may be at the organizational level of the cytoplasm and its inclusions.

Under the concept outlined above the analysis is most conveniently conducted by expressing all the relevant quantities (see p. 17) in their equivalent units of carbon. These quantities are:

(i) The change in the total carbon (designated ΔC).
(ii) The change in the carbon equivalent of the activity, (ΔCA).
(iii) The apparent specific activity of the carbon involved in the metabolic change during the period as a percentage of the specific activity of the C^{14}-source ($100\Delta CA/\Delta C$).

Before revealing how these quantities varied in the experiment, one can set down the type situations in which the metabolic change may seem to have been mediated by (i) carbon far richer in activity than its actual source, (ii) by carbon far less active than that supplied, (iii) by carbon of an infinitely high activity, or (iv) by carbon of an activity that appears to be negative. These type situations could be visualized as follows:

Situation (*i*), in which the carbon change seems to have been mediated by carbon which is far richer in activity than the actual source furnished. This situation could arise from concurrent disappearance of the inactive compound from one cellular phase, while it is increasing in the radioactive form in another.

Situation (*ii*), in which the change in the radioactive compound *A* seems to have been mediated by carbon which is far less active than the source. This situation could arise, not merely from the dilution of the entering compound by the same metabolite as it exists in the cell, but also by the concurrent appearance of the inactive compound being synthesized in one phase, while it is increasing in the radioactive form in another.

Situation (*iii*), in which the change in the radioactivity of the compound *A* seems to have been mediated by carbon of an infinitely high activity. This situation could arise when the total content of the metabolite in question, that is, the radioactive plus the inactive moieties, remains steady in the cell, and radioactive synthesis only occurs to replace the inactive compound *A* as it is converted to some other material. Under these circumstances there will be a progressive change in the radioactivity present in the compound *A* without a net increase in its total carbon. Thus radioactive synthesis will appear to have been achieved from a carbon source with infinite activity.

Situation (*iv*), in which it appears that the change in the amount of radioactivity present in the synthesized compound *A* seems to have been achieved by carbon whose activity has a negative sign. This anomalous situation would arise if the synthesis of the active compound *A* in one phase was accompanied by concurrent decrease in the inactive compound in another phase to such an extent that the *total amounts of the compound in the cell actually decrease during the period even though radioactive synthesis occurred.*

All these examples could be fitted plausibly into situations in which the C^{14}-labelled compound enters the cytoplasm from the outside solution and is converted into compounds which can exist in, and be subject to change in, *both* the vacuole and the mitochondria, to mention only two prominent types of cytoplasmic inclusion.

The foregoing clearly demonstrates the dangers inherent in considering only 'static' data—i.e. total activities, and even specific activities for a given time—from tracer experiments. It is very necessary to analyse the 'kinetic' data, after the manner indicated, to determine the direction and the amount of the change in the metabolites which occurred. This form of analysis may reveal situations which could never be disclosed by the analysis of more static data.

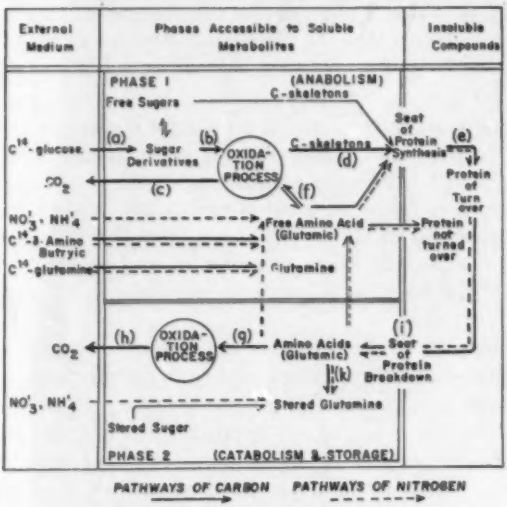
III. NITROGEN METABOLISM AND RESPIRATION OF CARROT TISSUE EXPLANTS AS REVEALED BY EXPERIMENTS WITH C^{14}-LABELLED SUBSTRATES

INTERPRETATION OF THE RESPIRING, SYNTHESIZING CELL IN TERMS OF SEPARATE PHASES OR COMPARTMENTS

A SCHEMATIC representation of the cell, Fig. 1, may now be introduced; this emphasizes the concepts which emerge from the data. The features of greatest interest are (i) the division of the cell into phases or compartments; (ii) the continuous breakdown and synthesis of protein and the production of carbon dioxide by oxidation of protein breakdown products; and (iii) the synthesis of proteins from carbon skeletons and nitrogenous groups which converge at the seat of synthesis. Several other points will be considered including the interconversion of such nitrogenous compounds as glutamine, glutamic acid, and γ-aminobutyric acid. The various pathways or metabolic routes in the

diagram have been labelled (*a*) to (*k*) for convenient reference. Only those metabolic routes which are required by the experimental data, or which are obviously necessary to complete the scheme (e.g. pathways of N) are shown. It must be stated, however, that the diagram is schematic, and does not attempt to assign the phases in question to such known morphological entities as vacuole, mitochondria, &c.

Diagram to Illustrate the Main Metabolic Pathways in Cultured Carrot Tissue Explants



Pathways emphasized by the experimental treatments are indicated by heavy lines

FIG. 1

Phases in cells. In Part II methods are developed by which data may be analysed in terms of separate phases or compartments, in which separate pools of a metabolite may undergo similar or divergent reactions without mingling. The main lines of evidence which establish such phases in the present experiment and lead to the assignment to them of various functions are as follows:

Tables IX and XI show that the specific activity of carbon in glutamic acid is nearly always very much greater than the specific activity of the carbon in glutamine, from which it was derived. In particular, when C^{14}-glutamine was supplied to fast-growing cultures, the specific activity of glutamic-acid-carbon was 99 at Period 1, and it remained above 80 during the entire experiment. This clearly indicates that glutamic acid could only have been derived directly from the glutamine supplied (specific activity = 100). In the same experimental sample, the specific activity of carbon in glutamine *in the tissue*

was only 66 at Period 1, dropping to the low level of 39 at Period 5. Table XIII also shows that the apparent specific activity of carbon in transit to glutamic acid was frequently, particularly in the early periods of the experiment, much greater than that of carbon in the source. These facts can be explained only if two pools of glutamine exist. One such pool gives rise to radioactive glutamic acid, and is replenished mainly from the radioactive glutamine in the external medium. In this manner the specific activity of the carbon in free glutamic acid is maintained at a high level. The other glutamine pool is mainly non-radioactive, and does not give rise to glutamic acid. Thus the specific activity of the carbon in the glutamine of the cell as a whole is low, while that of glutamic acid is high. It also follows that any non-radioactive glutamic acid formed in the cell (i.e. from protein breakdown or by synthesis from carbohydrate) must arise in a separate phase from the glutamic acid derived from glutamine supplied, and must be rapidly metabolized, since the specific activity of free glutamic acid carbon remains high.

When the tissue was furnished with radioactive glutamine it responded with a massive accumulation of glutamine (Table VIII). However, the specific activity of the carbon of the glutamine in the cell was initially (Period 1) somewhat lower than that of the source, and it decreased with time to only one-third that of the source (Period 5, Table XI). Table XIII shows that the specific activity of the carbon in transit to glutamine decreased continuously during the experiment. Thus, although radioactive glutamine was accumulating, inactive glutamine was accumulating much faster, and toward the end of the experiment nearly two-thirds of the total glutamine in the cell was inactive. A similar situation occurs in the tissues which were supplied with radioactive γ-aminobutyric acid (Tables VII, IX, and XIII). All the above data indicate that a large amount of glutamine arose from inactive carbon compounds. However, this non-radioactive glutamine could not have mixed with the radioactive glutamine which entered the cell, since the latter must have remained undiluted to supply the high specific activity of the carbon in free glutamic acid. Moreover, if the non-radioactive glutamine were formed from glutamic acid even in part, this glutamic acid could not have mixed with that formed from the glutamine which entered the cell from the medium.

It is possible now to consider the relationships of various compounds and functions in the cell. If the entry of radioactive glutamine into the cell and its conversion into glutamic acid occurs in that part of the cell which is designated Phase 1, then the formation and accumulation of any inactive glutamine which arises from protein must be located in a different part of the cell, namely Phase 2 (see Fig. 1). Moreover, since radioactivity from glutamine did enter the proteins (Table X), then the site of protein synthesis must be accessible to the glutamic acid of Phase 1, as shown in Fig. 1. Similarly, the seat of protein breakdown must be associated with Phase 2 in which the stored glutamine occurs.

It is evident that protein synthesis took place in all tissues, since radioactivity was found in protein amino-acids (Tables X, XII, and XV). However,

it seems probable that proteins were breaking down to supply the non-radioactive glutamine which accumulated when C^{14}-glutamine or C^{14}-γ-aminobutyric acid were supplied (no radioactive glutamine accumulated when C^{14}-glucose was supplied). This is consistent with the well-known idea of protein 'turnover'—the continuous synthesis and breakdown of proteins. It has been shown above that protein synthesis and breakdown must take place in separate phases of the cell (e.g. 1 and 2 of Fig. 1). It now remains to examine the part played in metabolism by protein turnover.

THE SUBSTRATES OF RESPIRATION

The data on the specific activity of the carbon in the supplied compounds and in the various constituents of the tissue, as well as the carbon dioxide produced, permit this general question to be examined (Tables III and IX to XV). The following points emerge:

(i) The specific activity of carbon dioxide progressively increased with time for all treatments, tending to be higher throughout in the fast-growing than the slow-growing tissues, but was not closely related to the activity of any of the soluble compounds of the tissue. When C^{14}-glucose was supplied, this quantity tended to follow the increasing specific activity of sugars *in the tissue* (Table XV). This was particularly evident in the fast-growing cultures supplied with C^{14}-glucose, when the increase in labelling of the sugars could largely account for the rise in specific activity of the carbon dioxide. Here the data strongly suggest that most of the carbon dioxide is formed by glycolysis and oxidation along routes *b* and *c* in Fig. 1. When C^{14}-glutamine or C^{14}-γ-aminobutyric acid were supplied, the specific activity of the carbon dioxide increased in a manner unrelated to that of the soluble compounds, and had no relation to the activity of sugars, which remained relatively unlabelled throughout (Tables III, IX, XI). Under these circumstances the labelled compounds may be drawn into oxidative processes along route (*j*) of Fig. 1.

(ii) Table III shows that emerging carbon dioxide had the highest relative specific activity when C^{14}-glucose was fed, and the lowest when γ-aminobutyric acid was supplied. The ability of the three substrates to label the carbon dioxide is given by the series glucose $>$ glutamine $>$ γ-aminobutyric acid in the ratio 4:2:1. As may be seen from Tables X, XII, and XV, these effects are paralleled by the similar order of labelling in the protein from the various radioactive substrates, the series again being glucose $>$ glutamine $>$ γ-aminobutyric acid, also in the ratio 4:2:1. Thus the degree of labelling of carbon dioxide was closely associated with that of protein; a common origin of carbon dioxide and of the carbon skeletons for protein synthesis is therefore indicated in glycolysis and oxidation processes of Phase 1 in Fig. 1.

(iii) Table XIV shows that the apparent specific activity of carbon entering protein amino-acids was sometimes greater than the specific activity of the source, or, if calculated, would be infinitely great. This must indicate that

radioactive amino-acids were being added to the protein while non-radioactive amino-acids were being removed. In other words, as previously noted, the protein was in a state of synthesis and breakdown, or turnover.

(iv) The high specific activity of respired carbon dioxide when labelled glucose was applied indicates that a large part of the respiratory substrate is derived, more or less directly, from the sugar of the medium. The parallelism between the specific activities of the sugars in the tissue and the carbon dioxide suggests that they have a common origin or that one is formed from the other. Since the specific activity of the carbon dioxide is sometimes higher than that of the free sugars of the tissues it cannot arise solely from them. A reasonable alternative, indicated in Fig. 1, is that the tissue sugars are formed from entering glucose through some intermediate which is also a main substrate of respiration. This is compatible with the idea that entering sugars are phosphorylated at the cell surface (Dormer and Street, 1949), or shortly after entering, and it is these sugar derivatives which subsequently give rise to free sugars in the tissues and, through the oxidative cycle, may lead to carbon dioxide (Vittorio, *et al.*, 1954). However, this cannot be the *only* pathway to carbon dioxide since the specific activity of carbon dioxide is appreciably lower than that of glucose in the medium (= 100).

(v) The scheme outlined in Fig. 1 recognizes the participation of glycolysis and of oxidative processes not only in cell respiration but also in the provision of intermediates for synthetic reactions. With particular reference to other plant materials, Yemm and Folkes (1954) and Willis and Yemm (1955) have discussed the possibility that a close coupling between carbohydrate and protein metabolism, operating in this way, may exist. High respiratory activity, generally associated with the synthesis of proteins and with other growth processes, may well result from regulatory mechanisms, involving oxidation-reduction and phosphorylation, which divert carbon skeletons to synthetic reactions. This, however, does not imply that conventional glycolytic and oxidative pathways are the *sole* routes to carbon dioxide in cell metabolism. The results now under discussion suggest that growth and the rapid synthesis of proteins may superimpose other metabolic and respiratory pathways upon those of the resting cell and thus lead to new concepts of the mechanisms of cell metabolism. Nevertheless, it should not be concluded that the suggested mechanisms act solely or exclusively. In a dynamic cellular system with the degree of specialization in its compartments or organelles that is here contemplated, it is unlikely that one pathway operates to the complete exclusion of others. The purpose of this discussion is rather to emphasize that conventional interpretations in terms of glycolysis and associated oxidative cycles may be inadequate to explain all the facts of respiration and metabolism, particularly in growing cells.

All the above facts, taken together, clearly indicate that there are two main pathways by which carbon dioxide may emerge. In one pathway, presumably compatible with conventional glycolysis, Krebs cycle or other well-known oxidative systems, carbon dioxide is derived from sugars, or sugar derivatives,

via routes *a*, *b*, and *c* of Fig. 1. Carbon dioxide could also be derived from the supplied nitrogen compounds through their interaction with the Krebs cycle as at (*i*) of Fig. 1. In the other pathway, carbon dioxide may be derived from products of protein breakdown, following the routes *a*, *b*, *d*, *e*, *f*, *g*, and *h* in Fig. 1. This second route to carbon dioxide is consistent with the relationship between the labelling of the carbon in the protein and in the carbon dioxide, and is also compatible with the absence under some conditions of a relationship between labelling in the soluble compounds and the carbon dioxide. The idea of protein turnover, and the failure of protein breakdown products to accumulate (except as glutamine), as noted above, also supports the idea expressed in the route *a* to *h* on Fig. 1. The increasing specific activity of carbon dioxide with time, as noted at (i) above, requires that carbon dioxide may emerge from a source which is initially unlabelled but which acquires label steadily during the experiment. This source may be carbohydrate; it may also in part be protein.

SUBSTRATES OF PROTEIN SYNTHESIS

It has been shown above that the proteins may be a source of carbon dioxide, in that their breakdown products are used in respiration. In this way, radioactive carbon could appear in carbon dioxide late in the experiment, after its passage through proteins. It is now of interest to inquire how that radioactivity entered the protein.

(i) When C^{14}-glucose was supplied, the protein amino-acids achieved a high level of activity, much higher than when C^{14}-γ-aminobutyrate or glutamine were supplied (Tables X, XII, and XV).

(ii) When C^{14}-glutamine was supplied, free glutamic acid at once attained a very high specific activity, which it maintained throughout the experiment (Table XI). However, the specific activity of protein glutamic acid was not high (Table XII), being not much different from that of other protein amino-acids, and much lower than when glucose was supplied (Table XV).

When γ-aminobutyric acid was supplied, essentially the same result emerged, for glutamic acid had the highest specific activity after the compound supplied (Table IX). Again, this was not reflected in the protein glutamic acid, whose specific activity was actually *lower* than that of other protein amino-acids (Table X).

In general, from Tables IX to XII and XV, the distribution of activity in protein amino-acids does not reflect directly the activity in the free amino-acids of the tissues.

(iii) Tables X and XII show that, when labelled nitrogen compounds were supplied, the specific activity of several protein amino-acids, notably glutamic and aspartic acids, hydroxyproline and, at times, proline, increased steadily and all these amino-acids behave in the same way. Surprisingly, alanine in the protein acquired little or no label, except when C^{14}-glucose was supplied (Table XV), in spite of the fact that free alanine became strongly radioactive

from C^{14}-glutamine (Table XIII). Once again it must be stressed that the distribution of activity among the protein amino-acids does not reflect that of the free amino-acids. However, there is a remarkable similarity among certain of the combined amino-acids in the rate and the degree of labelling that they acquire.

The above points together provide strong evidence that in the carrot cultures, glucose is the direct source of carbon for proteins. Moreover, the carbon from glucose must be incorporated into the protein by a direct route, not involving its prior equilibration with or conversion to free amino-acids. Glucose carbon was more readily converted to protein than glutamine carbon and labelled glutamic acid; thus conversion of glucose to glutamic acid must have occurred. Since (*a*) there was no dilution of the specific activity in the free glutamic acid which arose from supplied glutamine, and (*b*) neither was the high degree of labelling in free glutamic acid reflected in the protein, therefore the glutamic acid formed from glucose *en route* to protein could never have been free. In this situation one may visualize that carbon skeletons arising from glucose metabolism via *a*, *b*, and *d* in Fig. 1 could combine with nitrogenous groups at the seat of protein synthesis. Any amino-acids so formed could have but a fleeting existence, being converted promptly to protein; indeed the entire process of amination and condensation might well be simultaneous.

It follows that, if a labelled substance which is associated with the Krebs cycle is supplied to the tissue (e.g. glutamic acid arising from the C^{14}-glutamine and C^{14}-γ-aminobutyric acid supplied), its carbon may enter proteins by entering the Krebs cycle (as at *i* of Fig. 1). This carbon becomes indistinguishable from the carbon from the metabolism of glucose, as it is converted to carbon skeletons, which give rise to protein (see *d* of Fig. 1). Thus all the carbon skeletons emerging from the Krebs cycle (as at *d* of Fig. 1) would be labelled to the same extent, and protein amino-acids formed from them would acquire label to the same extent and at the same rate. This, in fact, occurred.

The exceptional behaviour of alanine bound in the protein, which became strongly labelled from C^{14}-glucose, but was unlabelled from C^{14}-glutamine, even though free alanine became radioactive, can now be explained. If the alanine in protein arose from pyruvic acid, as the carbon skeleton, and some nitrogen donor, it would be expected to be strongly radioactive when C^{14}-glucose was supplied. However, when C^{14}-glutamine was supplied, its activity would not be expected to enter pyruvic acid to any extent (Bidwell, *et al.*, 1955).

There is a further major point of evidence in support of the scheme at Fig. 1. Whenever the total protein synthesis is emphasized, as for example by the use of coconut milk which stimulated growth by cell division in the fast-growing cultures, the metabolic pathways that involve protein synthesis and breakdown also tend to be emphasized. This point will be discussed more fully in Part IV of this paper, which will deal with the impact of growth on the metabolism of cells.

SUMMARY OF THE OVERALL METABOLIC SCHEME AS PRESENTED

In broad summary, the following essential points have been stressed. The cells in these tissues present a system in which there are isolated pools of metabolites, here called Phases. The metabolites in these phases may be merely storage products, or they may be involved in a variety of reactions, according to the accessibility of various reaction sites. The immediate precursors of proteins associated with the seat of protein synthesis arise in one phase, while the products of protein breakdown are metabolized in another.

In these tissues there are two main pathways for the oxidation of carbon compounds. One path proceeds via carbohydrates and the Krebs cycle direct to carbon dioxide. The other path stems also from carbohydrates via the Krebs cycle, moves through carbon receptors of nitrogen into protein and thence, via protein breakdown and oxidation of the breakdown products, reaches carbon dioxide. It is also evident that most of the free amino-acids of the tissue are not the immediate precursors of protein at the seat of synthesis nor do they readily equilibrate with them. It is necessary to visualize some form of condensation by which the donors of nitrogen and the carbon residues, with the necessary energy, are converted directly to proteins. This occurs in a part of the cell which is remote from the soluble reserves of amino-acids.

INTER-RELATIONSHIPS BETWEEN NITROGENOUS COMPOUNDS

The data from the experiment permit the relationships between various nitrogen compounds to be examined.

γ-aminobutyric acid, glutamic acid, and glutamine. From its first discovery in higher plants (Steward, Thompson, and Dent, 1949), there has been an idea that γ-aminobutyric acid arises as a decarboxylation product of glutamic acid. While this is feasible, it requires substantiation. Alternatively, γ-aminobutyric acid might arise in some other way, as, for example, from succinic semi-aldehyde, and be carboxylated to give glutamic acid. The evidence on these points can be extracted from Tables IX to XIII, from which it is possible to calculate the amount of glutamine and glutamic acid which was derived from the supplied γ-aminobutyric acid, and also the amount of γ-aminobutyric acid derived from the supplied glutamine.

To account for the formation of glutamine and glutamic acid from radioactive γ-aminobutyric acid, the appropriate correction was made to allow for the dilution with the inactive carbon that would be involved in the carboxylation reaction. When γ-aminobutyric acid was furnished this constituted its sole source to the tissue; there was obviously no endogenous production since its specific activity remained consistently very high. Over the whole period of the experiment the ten slow-growing explants synthesized 45 μg. of carbon in the form of glutamine and glutamic acid from γ-aminobutyric acid, and the fast-growing cultures synthesized 71 μg. (calculated from Table XIII). In

the corresponding treatment with C^{14}-glutamine there was some synthesis of γ-aminobutyric acid. In the fast-growing cultures there was a net synthesis of 20 μg. of γ-aminobutyric acid during the whole period, of which only 6·7 μg. came from the radioactive glutamine. In the slow-growing cultures, on the other hand, the net synthesis was of the order of 0·6 μg., all of which could be accounted for from the glutamine (Table XIII). The inactive glutamine, shown to arise in Phase 2 from protein breakdown, could also give rise to inactive γ-aminobutyric acid; this, however, may be neglected when estimating the turnover of glutamine to γ-aminobutyric acid based on radioactive labelling since the two glutamine pools were completely separate. Hence, 10 slow-growing explants supplied with radioactive glutamine manufactured 0·67 μg. of γ-aminobutyric acid and 10 explants of the fast-growing tissue synthesized 6·7 μg. These amounts are to be compared with the quantities of 45 and 71 μg. of glutamine and glutamic acid which arose from γ-aminobutyric acid in similar tissue. The amounts of glutamine and glutamic acid derived from γ-aminobutyric acid were probably very much larger than here calculated, due to the great metabolic activity of these two compounds.

Therefore, in the slow-growing tissue the conversion of γ-aminobutyric acid to glutamine and glutamic acid was at least 70 times faster than the conversion of glutamine to γ-aminobutyric, and in the faster growing tissue the conversion of γ-aminobutyric acid to glutamine and glutamic acid was at least 70 times faster than the converse reaction. These differences in the ease of conversion of γ-aminobutyric acid to glutamine and of glutamine to γ-aminobutyric acid are so striking that they may be seen on radioautographs which represent equivalent amounts of tissue, as illustrated in Fig. 2. This shows that when γ-aminobutyric acid (No. 23) was supplied, glutamine (No. 9) was at times (slow-growing tissue) present in excess of the compound supplied. However, when glutamine was supplied, γ-aminobutyric acid was hardly detectable, though pyrrolidone carboxylic acid (No. R3) was conspicuous.

The important conclusion from these observations is that γ-aminobutyric acid is now to be regarded much more as a potential source of glutamic acid and of glutamine than as a product arising from them by decarboxylation. This presupposes an alternative route for the biogenesis of γ-aminobutyric acid, and the obvious precursor is succinic semialdehyde. Parenthetically, it may be noted that another tissue, potato tuber, fixes carbon dioxide in the dark almost exclusively in glutamine; this is consistent with carboxylation of γ-aminobutyric acid (Steward and Pollard, 1956).

Glutamic acid could be an intermediary between γ-aminobutyric acid and glutamine; evidence for this is in Table IX. In all cases but one, including both slow- and fast-growing explants furnished with C^{14}-γ-aminobutyric acid, the specific activities of the substances in the tissue are in the order γ-aminobutyric acid > glutamic-acid > glutamine. Therefore, glutamic acid may be an intermediary, although this is not proved.

Table XIII gives the specific activity of the carbon *in transit* through

glutamic acid after supply as γ-aminobutyric acid, and shows that the specific activity of the carbon moving through glutamic acid is consistently less than the specific activity of the carbon in the γ-aminobutyric acid; this indicates that there are other sources than γ-aminobutyric acid for the replenishment of the glutamic acid pool. This is, of course, to be expected.

Before leaving the relations of glutamine and its related compounds, reference should be made to the substance labelled R3 on Fig. 2. This is chromatographically identical with pyrolidone carboxylic acid. The striking intensity of R3 in the tissue extracts from the treatments with C^{14}-glutamine raises the possibility that this substance is produced metabolically since the intensity observed seems to be in excess of that which could be due to equilibration during the extraction.

Aspartic acid and asparagine. Both glutamic acid and glutamine transfer their radioactivity to asparagine and to aspartic acid in the protein (Tables IX to XII). (Free aspartic acid was obscured on the chromatograms by some radioactive but ninhydrin inactive unknown substances, R4.) However, Tables X and XII show that aspartic acid of the protein became radioactive at the same rate and to the same extent as glutamic acid when either γ-aminobutyric acid or glutamine were supplied as the radioactive source, and free asparagine behaved in approximately the same manner (Tables IX, XI). This points to the incorporation of aspartic acid in the protein via a similar pathway as glutamic acid, namely via the Krebs cycle (route *j, d, e* in Fig. 1). Thus labelled glutamic acid enters the Krebs cycle and is there diluted by carbon from sugars. Carbon skeletons, ultimately forming the aspartic and glutamic acids of proteins as well as free aspartic acid and asparagine, are provided at the same level of specific activity. This appears to fit well with the observed facts and theory as explained earlier in connexion with the scheme represented by Fig. 1.

Proline and hydroxyproline. It is a familiar fact of nitrogen metabolism that proline is derived from glutamic acid. Although hydroxyproline occurs with some frequency in plant proteins, its occurrence in the free state is comparatively rare, though examples have been noted of its occurrence free in considerable quantity (Steward, Thompson, and Pollard, 1957). The proteins of the growing-tissue cultures are of interest because they contain hydroxyproline in far greater amount than the protein of the tissue of the intact organ from which they were derived. There are, therefore, these additional features of interest in the consideration of the interrelationships of glutamine, proline, and hydroxyproline in the soluble and the protein fractions respectively. The recent discovery of hydroxyglutamic acid as a free constituent of plants (Virtanen and Hietala, 1955; Fowden and Steward, 1956) suggests the possibility that this substance could lead to hydroxyproline in somewhat the same fashion that glutamic acid gives rise to proline.

When γ-aminobutyric acid and glutamine labelled with C^{14} were supplied, proline and hydroxyproline were readily labelled in the protein (Tables X and XII). In fact, *after the amino-acids glutamic and aspartic acid these were the*

most highly labelled compounds in the protein. Also, the proline which occurred in the free state was readily labelled from these sources, although hydroxyproline was generally absent (Tables IX and XI). Free proline had a higher specific activity than the proline and hydroxyproline combined in the protein, so that it could have given rise to both these substances. However, since hydroxyproline does not occur free, it may be that it only arose from proline after embodiment in the protein. The general impression from the recorded data is that these compounds are closely connected with the nitrogen compounds supplied, being synthesized from them rather directly. This is in contrast to some observations made on other plants (Bidwell, *et al.*, 1955).

Observations on alanine and threonine. As shown in Tables IX to XII, XV, and Fig. 2, alanine (No. 8) was not radioactively labelled from the γ-aminobutyric acid source, was moderately labelled when radioactive glutamine was supplied, but was strongly labelled when radioactive sugar was furnished. There are precedents for the ease of labelling of alanine via glucose, in contrast to the comparative difficulty of labelling from glutamic acid or glutamine (Bidwell, *et al.*,1955). These observations agree with the conventional idea that alanine arises from pyruvic acid and therefore that carbon should enter alanine after prior incorporation in the Krebs cycle from glucose, i.e. via *a*, *b*, *d*, *e*, of Fig. 1. These observations were based on the alanine as it exists in the protein, since the measurements of specific activity of the alanine in the free state were complicated by the spread of radioactivity from glutamine over the alanine spot on the chromatograms.

From the ease of labelling combined threonine and alanine the following observations can be made. When γ-aminobutyric acid was supplied to the tissue alanine did not become appreciably labelled whereas threonine did. Furthermore, when glutamine was supplied the specific activity of carbon in threonine was greater than that in alanine wherever they could be compared in the same extract. Threonine did become labelled from radioactive sugar, but not so intensely as alanine. The conclusion is that alanine lies more nearly athwart the line of synthesis from sugar to protein, whereas threonine lies more closely alongside the metabolism of glutamine, proline, and hydroxyproline.

THE FULL RANGE OF COMPOUNDS THAT ACQUIRED RADIOACTIVITY FROM THE SUBSTANCES SUPPLIED

The preceding tables have presented the data which relate to those compounds that acquired radioactivity in sufficient degree to be measurable. It is now desirable to indicate the complete range of substances into which radioactivity entered, even though in many of these the intensities were small. In addition to the ninhydrin reactive substances and the sugars a number of other, as yet unidentified, substances appeared on the radioautographs; their positions were recorded and they have been designated by a series of symbols (R1–R9, Fig. 2). The characteristics of these substances, so far as they are known, are listed in Table XVI. The full list of the substances that became

TABLE XVI

Unidentified radioactive substances produced from the C^{14}-labelled substrates supplied

Designation	R_f phenol	R_f collidine-lutidine	Notes on properties of the substance
Alanine	0·585	0·287	Reference R_f.
R1	0·78	0·41	—
R2	0·94	0·41	—
R3	0·63	0·30	Strong spot C^{14} glutamine supplied, probably pyrrolidone carboxylic acid.
R4	0·10	0·13	Complex of 3 possibly distinct spots.
R5	0·80	0·22	—
R6	0·40	0·25	—
R7	0·62	0·44	—
R8	0·83	0·48	—
R9	0·23	0·19	—

radioactive under these various treatments is shown in Table XVII, on which the following general comments may be made.

It is quite conspicuous, especially in the slow-growing cultures, that there was very little labelling of the soluble amino-acids from glucose despite the fact that a wide range of amino-acids was labelled in the protein. This bears out the interpretation that the carbon framework for protein synthesis comes from glucose and not directly from the soluble amino-acids of the tissue. Conversely, the carbon supplied in the form of glutamine and γ-aminobutyric acid was readily dispersed through a very wide range of the soluble compounds including amino-acids (see Fig. 2). Due to the fact that the specific activity of the nitrogen compounds supplied was high the activity appeared in a large number of amino-acids in the protein but most of these represented only a small percentage of the total activity supplied, in contrast to the activity which appeared in the amino-acids of the protein when labelled sugar was supplied.

Some other more detailed relationships may be noted. When γ-aminobutyric or glutamine was the source the basic amino-acids arginine, histidine, and lysine became labelled in the protein, but, when glucose was the source, arginine and histidine were invariably unlabelled and only in one case was lysine labelled.

Glycine, which is invariably absent from the soluble fraction, became radioactive, in all cases but one, in the protein.

Serine, in the protein, invariably became radioactive from the glucose but this did not occur when the carbon was furnished in the form of γ-aminobutyric acid and glutamine. The serine never became radioactive in the soluble fraction, even when it did so in the protein.

Tyrosine became radioactive in the protein of the slow-growing cultures in all cases, but was only radioactive in the protein of the fast-growing cultures when glucose was furnished.

In summary, it is a general observation that a larger number of amino-acids became labelled in the protein than in the soluble fraction (this is especially so when C^{14}-labelled glucose was supplied). This supports the view that it is a carbon precursor which is condensed with a nitrogen donor to form protein

TABLE XVII

Compounds which acquired radioactivity when carrot tissue explants, both slow growing (no C.M.) and fast growing (+C.M.), were supplied with C^{14}-labelled glucose, γ-aminobutyric acid, or glutamine. (S = soluble, I = insoluble fractions)

Compound supplied	C^{14}-glucose				C^{14}-γ-amino-butyric acid				C^{14}-glutamine			
	no CM		+CM		no CM		+CM		no CM		+CM	
Fraction	S	I	S	I	S	I	S	I	S	I	S	I
Compounds:												
Aspartic acid		+	+	+	+	+	+	+	+	+	?	+
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+
Asparagine					+		+		+		+	
Glutamine					+		+		+		+	
Serine		+		+						?		
Glycine		+		+		+				+		+
Threonine		+		+	+	+	+	+	+	+	+	+
Alanine		+	+	+	+	+	+	+	+	+	+	+
γ-aminobutyric acid					+		+		+		+	
Lysine				+		+		+		+		+
Arginine						+		+		+	?	+
Histidine						+		+		+		+
Tyrosine		+		+		+				+		
Valine	+	+		+	+	+	+	+	+	+	+	+
Phenylalanine												
Leucines		+		+	+	+	+	+		+	+	+
Proline		+	+	+	+	+	+	+	+	+	+	+
Hydroxyproline		+		+	+	+	+	+		+	+	+
Glucose+sucrose	+		+		+		+		+		+	
Fructose	+		+		+				+			
R1					+		+		+		+	
R2	+		+		+		+		+		+	
R3							+		+	+	+	
R4	+		+		+		+		+		+	
R5							+					
R6					+		+		+		+	
R7	+			?					+		+	
R8					+		+		+		+	
R9		+	+									
R9a			+									

and not, specifically, the free amino-acids. As suggested for hydroxyproline, other hydroxy-amino-acids (serine, threonine, tyrosine) may be formed after incorporation in the protein.

The nine unidentified radioactive areas on the chromatograms may be classified as follows.

Four of these, namely R1, R3, R6, and R8, only appeared when the radioactive substance supplied was a nitrogen compound, namely γ-aminobutyric,

or glutamine. One of these (R3) is believed to be pyrrolidone carboxylic acid, probably derived metabolically from glutamine. One unknown (R9) seems to be peculiar to the tissue treated with glucose; R2 and R4 occurred in all the tissues; R7 appeared after treatment with radioactive glucose and glutamine, and R5 was only noticed in one instance.

Parts IV and V of this group of papers are to follow. These will deal first with an analysis of the impact of growth on the fresh weight, protein metabolism, and respiration of carrot tissue, as shown by the data presented, and then with a general discussion of the results of, and problems raised by, these experiments. Pending this further analysis and more general discussion the main conclusions from Parts I, II, and III have been indicated in the text and they are briefly stated in the summaries that precede the text and presentation of the data.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge that the supplies of the radioactive substrates were furnished due to the interest shown by Dr. G. Krotkov in these investigations. Discussion with Dr. Krotkov occurred at the planning stage.

One of us (E. W. Y.) was able to work in the Botany Department, Cornell University, during a period of tenure of a Rockefeller Foundation Fellowship.

The special facilities for, and assistance with, the work on tissue cultures were furnished under a grant to one of us (F. C. S.) from the National Cancer Institute, National Institutes of Health, of the Department of Health, Education and Welfare. In particular the assistance of Miss K. Mears is especially acknowledged.

In the use of chromatography and radioautography additional help was needed. This was furnished by Mr. D. Margolis, Mrs. Ruth Greenwood, and especially Dr. J. K. Pollard, all of these working with one of us (F. C. S.).

To the Laboratory at Kingston, through one of us (R. G. S. B.), we owe all the measurements of radioactivity used in the calculation of specific activities.

For all this help we express our thanks.

DESCRIPTION OF PLATE

FIG. 2. Radioautographs of chromatograms of alcohol soluble fraction of slow and fast growing carrot tissue explants showing the effects of C^{14}-labelled substances supplied.

Key: 2 = aspartic acid; 3 = glutamic acid; 6 = asparagine; 7 = threonine; 8 = alanine; 9 = glutamine; 15 = proline; 16 = valine; 23 = γ-aminobutyric acid; 24 = hydroxyproline. R3 = pyrrolidone-carboxylic acid. F = fructose; G & S = glucose and/or sucrose.

R1, R2, R4, R6, R7, and R8 = radioactive, ninhydrin nonreactive unidentified substances.

LITERATURE CITED

BIDWELL, R. G. S., KROTKOV, G., and REED, G. B. (1954). Synthesis of radioactive glutamine from $C^{14}O_2$ in swiss chard leaves and its isolation by paper chromatography. *Arch. Biochem. Biophys.* **48**, 72–83.

—— —— —— (1955). The influence of light and darkness on the metabolism of radioactive glucose and glutamine in wheat leaves. *Canadian J. Bot.* **33**, 189–96.

—— VITTORIO, P. V., KROTKOV, G., and REED, G. B. (1955). The effect of penicillin on CO_2 production and the uptake of C^{14}-labelled glucose and glutamine by *Staphylococcus aureus*. *Canadian J. Microbiol.* **1**, 634–43.

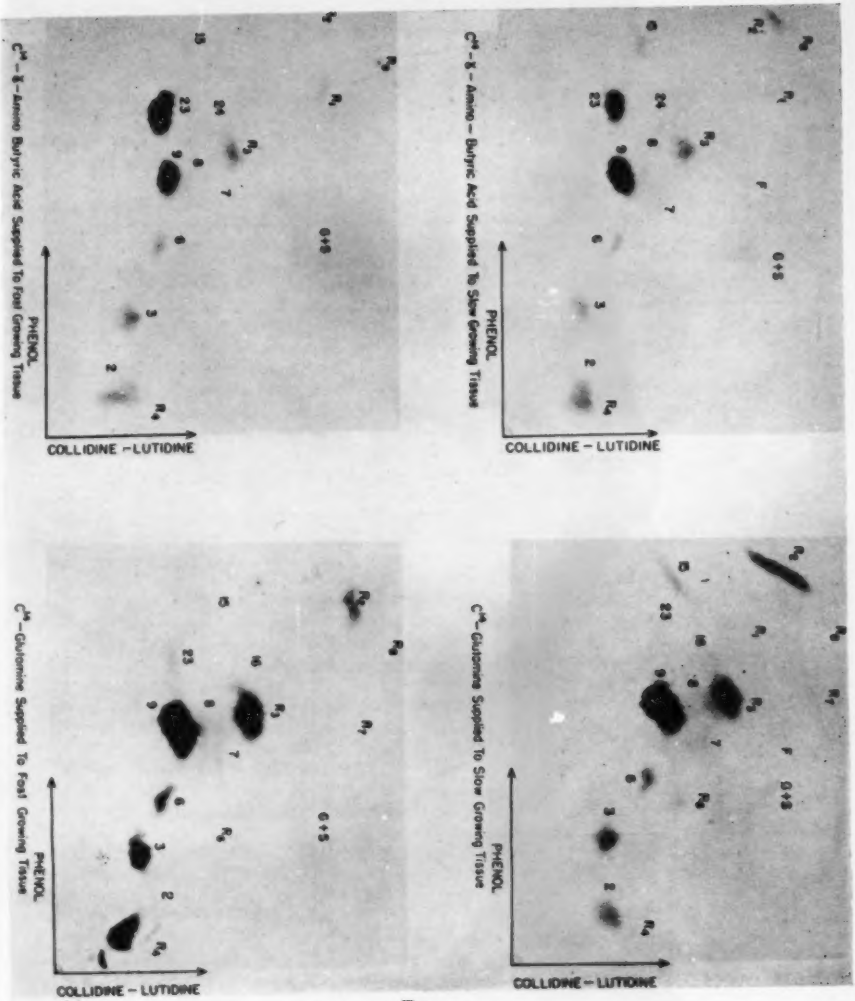


FIG. 2

F. C. STEWARD, R. G. S. BIDWELL *and* E. W. YEMM—PLATE I

CALVIN, M., HEIDELBERGER, C., REID, J. C., TOLBERT, B. M., and YANKWICH, P. F. (1949). *Isotopic Carbon*, John Wiley & Sons, Inc., New York.

CAPLIN, S. M., and STEWARD, F. C. (1949). A technique for the controlled growth of excised plant tissue in liquid media under aseptic conditions. *Nature*, **163**, 920.

DORMER, K. J., and STREET, H. E. (1949). The carbohydrate nutrition of tomato roots. *Ann. Bot.* N.S. **13**, 199–215.

DREYWOOD, R. (1946). Qualitative test for carbohydrate material. *Jour. Ind. Eng. Chem. Anal. Ed.* **18**, 499–500.

FOWDEN, L., and STEWARD, F. C. (1956). Nitrogen compounds and nitrogen metabolism in the Liliaceae. I. The occurrence of soluble nitrogen compounds. *Ann. Bot.* **21**, 53–67.

NELSON, C. D., and KROTKOV, G. (1955). Specific activities of carbon counted in a methane-flow proportional counter either as organic carbon or as barium carbonate. *Arch. Biochem. Biophys.* **59**, 294–5.

PARTRIDGE, S. M. (1946). Application of the paper partition chromatogram to the qualitative analysis of reducing sugar. *Nature* **165**, 270–1.

STEWARD, F. C., BIDWELL, R. G. S., and YEMM, E. W. (1956). Protein Metabolism, Respiration and Growth: A Synthesis of Results from the use of C^{14}-labelled substrates and tissue cultures. Ibid. **178**, 734–8; 789–92.

—— CAPLIN, S. M., and MILLAR, F. K. (1952). Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. *Ann. Bot.* N.S. **16**, 58–77.

—— —— and SHANTZ, E. M. (1955). Investigations on the growth and metabolism of plant cells. V. Tumorous growth in relation to growth factors of the type found in coconut. Ibid. **19**, 29–47.

—— and POLLARD, J. K. (1956). Some further observations on glutamyl and related compounds in plants, in *Inorganic Nitrogen Metabolism*, W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore.

—— and SHANTZ, E. M. (1956). The chemical induction of growth in plant tissue cultures, in *Symposium on Growth Regulating Substances*, E. L. Wain and F. Wightman, Butterworth's, London.

—— and THOMPSON, J. F. (1954). Proteins and protein metabolism in plants, in *The Proteins*, Ed. H. Neurath and K. Bailey. II A pp. 513–94, Academic Press Inc., N.Y. See Table 13, p. 537.

—— ——, and DENT, C. E. (1949). γ-Aminobutyric acid: a constituent of potato tuber? *Science* **110**, 439–40.

—— ——, and POLLARD, J. K. (1957). Contrasts in the nitrogenous composition of rapidly growing and non-growing plant tissue. *J. Exp. Bot.* (in press).

THOMPSON, J. F., POLLARD, J. K., and STEWARD, F. C. (1953). Investigations of nitrogen compounds and nitrogenous metabolism in plants. III. γ-Aminobutyric acid in plants, with special reference to the potato tuber and a new procedure for isolating amino acids other than γ-amino acids. *Plant Physiol.* **28**, 401–14.

—— and STEWARD, F. C. (1951). Investigations of nitrogen compounds and nitrogen metabolism in plants. I. The reaction of nitrogen compounds with ninhydrin on paper: a quantitative procedure; II. Variables in two directional paper chromatography of nitrogen compounds: a quantitative procedure. Ibid. **26**, 375–97 and 421–40.

TURNER, S. J. (1940). Respiratory metabolism of carrot tissue. III. The drift of respiration and fermentation in tissue slices with notes on the respiratory quotient. *Aust. Jour. Biol. Med. Sci.* **18**, 273–98.

VIRTANEN, A. I., and HIETALA, P. K. (1955). γ-Hydroxyglutamic acid in green plants. *Acta. Chim. Skand.* **9**, 175–6.

VITTORIO, P. V. (1953). *The use of Carbon-14 in the study of starch and sugar synthesis in detached tobacco leaves*. Ph.D. Thesis, Queen's University, Kingston, Ontario.

—— KROTKOV, G., and REED, G. B. (1954). Synthesis of radioactive sucrose by tobacco leaves from C^{14}-uniformly labelled glucose and glucose -1- phosphate. *Canadian J. Bot.* **32**, 369–77.

WHITE, P. R. (1943). *A Handbook of Plant Tissue Culture*, Jaques Cattell Press, Lancaster, Pa.

WILLIS, A. J., and YEMM, E. W. (1955). The respiration of barley plants. VIII. Nitrogen assimilation and the respiration of the root system. *New Phytol.* **54**, 163–81.

YEMM, E. W., and FOLKES, B. F. (1954). The regulation of respiration during assimilation of nitrogen in *Torulopsis utilis*. *Biochem. J.* **57**, 495–508.

Fat Metabolism in the West African Oil Palm (*Elaeis guineensis*)

PART 2. FATTY ACID METABOLISM IN THE DEVELOPING SEEDLING

S. G. BOATMAN[1] AND W. MARY CROMBIE
University of Southampton

Received, 18 May 1957

SUMMARY

During germination in the light, the endosperm, containing a high proportion of reserve fat (composed largely of shorter-chain (C_8 to C_{14}) saturated fatty acids), is slowly invaded by the expanding haustorium (cotyledon). Free fatty acids accumulate in the endosperm, preferential hydrolysis of longer-chain saturated acids (C_{14} to C_{18}) occurring under conditions of slow growth. Lipids are absorbed by the haustorium, the process being superficially similar in certain respects to intestinal fat absorption. When complicating factors are removed, absorption is found to be unselective during disappearance of 75 per cent. of the endosperm lipids.

Amounts of lipid in the haustorium are low compared with the high concentration in the surrounding endosperm and, before photosynthesis starts, losses through respiration account for a large part of the reserves which disappear. No free fatty acids are present in the haustorium.

Breakdown of fatty acids is relatively unspecific, although the acids characteristic of the haustorium (C_{18}, C_{18}, oleic and linoleic acids) are metabolized somewhat less rapidly than the shorter-chain saturated acids (C_8 to C_{14}) characteristic of the endosperm fat.

Both root and shoot have a low fat content. The fatty-acid composition of the former changes little during growth, but in the shoot linolenic acid increases proportionately during leaf expansion in the light.

FAT formation has already been described in oil-palm kernels (Part 1) and the present work was undertaken to compare fat synthesis and utilization in the same organ. Breakdown of the endosperm in *Elaeis* involves development of an absorptive cotyledonary structure, the haustorium, and separate analyses have been made of endosperm, haustorium, root and shoot at different stages of development.

Elaeis contrasts with *Citrullus* which has already been examined (Crombie and Comber 1956) for two reasons: first it is a seed with endosperm reserves, and second, the nature of the fat in the two genera is different (highly saturated in *Elaeis*, highly unsaturated in *Citrullus*).

MATERIALS

West African Oil Palm seed germinates both slowly and erratically, and it was therefore impracticable to obtain the large number of seedlings required

[1] Present address: The Rubber Research Institute of Malaya, Kuala Lumpur, Malaya.

by germination in Southampton. Instead a large uniform sample of just-germinated nuts (var. dura) was sent by air from Nigeria and planted in sand in a greenhouse at 27° C. (maximum fluctuations 16°–52°), with 12-hour fluorescent lighting supplementing daylight. Such seedlings developed normally and were grown successfully for more than a year, extra minerals being supplied after some months by watering weekly with modified Knops culture solution.

Duplicate random samples (50 nuts each) were taken on arrival (0 days) and at 20, 40 90, and 160 days after planting out. In addition two samples of ungerminated nuts were obtained separately from Nigeria. Any nuts containing more than one kernel were rejected.

After removal of the hard endocarp, each sample was divided into shoot, roots, 'endosperm' (including testa), and haustorium, for separate analysis of these parts (in the ungerminated stage, the whole seed was treated as 'endosperm', and in the 0-day stage root and shoot had not developed sufficiently to be divided). Particular care was taken to separate the haustorium from adhering droplets of semi-liquid endosperm by washing it carefully with water. This technique was established as satisfactory, since no extra fatty material was removed in a control experiment by subsequent washing with acetone followed by light petroleum (Boatman, 1956).

ANALYTICAL METHODS

Each sample was plunged into boiling acetone for 10 mins.; it was then stored in acetone for subsequent analysis.

The methods of analysis for dry weight, light petroleum (b.-pt. 40°–60°) extract, carbohydrates (starch, sucrose, and reducing sugars) and examination of the lipid extract are already fully described (Crombie and Boatman, 1955; Crombie, 1956; Crombie and Comber, 1956). Micro-hydrogenation of fatty acids dissolved in glacial acetic acid, over Adams platinum oxide catalyst, followed by chromatography, was used as an additional analytical method (Boatman, 1956). Normally, acid percentages determined by the chromatographic technique, are calculated from the difference between total (saturated and unsaturated) and oxidized (saturated only) fatty acids. In a few cases percentages were also calculated from the difference between total and hydrogenated acids: these are indicated in the tables by square brackets.

Free volatile fatty acids were determined by Method Cd 5–40 of the American Oil Chemists' Society 1953, and volatile combined acids were estimated by the same method on an acidified sample of the soaps produced by saponification (Boatman, 1956). Hydroxyl values were estimated by method Cd 4–40 of the American Oil Chemists' Society 1953.

RESULTS

1. *Morphology and anatomy*. A small straight embryo is embedded at one side of the solid fatty endosperm. The endosperm itself is surrounded by a closely adhering thin testa, and the whole is enclosed in a hard endocarp with

a germ pore adjacent to the embryo. On germination the embryo enlarges and forces its way out through the germ pore, to form externally a small button of tissue containing root and shoot initials. At the same time the other end of the embryo enlarges to form a cotyledonary structure, the haustorium, which starts to absorb the endosperm (Fig. 1). As development proceeds the endosperm is progressively digested and the haustorium enlarges until it eventually

FIG. 1. Progressive digestion of the endosperm by the haustorium during germination. The hard endocarp has been removed and the haustorium is shown stippled.
A, ungerminated nut; B, 0 days; C, 20 days; D, 40 days; E, 90 days; F, 160 days.

fills the seed cavity; finally the haustorium rots away and the seedling becomes independent (Yampolsky, 1922; Henry, 1951; Boatman, 1956; Anon. 1956).

During germination the endosperm immediately surrounding the haustorium softens, apparently owing to dissolution of the middle lamellae and cellulose cell-wall thickenings. The cell contents, consisting chiefly of solid fat and oily droplets, become disorganized and are slowly absorbed leaving a layer of crushed walls, which are also eventually broken down and absorbed by the haustorium. At the 90-day stage of the present experiment only a few layers of endosperm cells remained intact, while by 160 days only a single layer of these cells remained within the testa.

The haustorium is an ovoid spongy organ composed mainly of aerenchymatous tissue; the outer layers of cells are more compact and the highly convoluted surface is covered by a columnar epithelium, whose cells contain dense cytoplasm. There is an extensive vascular supply connecting this organ with both root and shoot.

Microchemical tests on hand sections of the haustorium disclosed that, in the early germination stages, the epidermal cells, and those immediately within them, contain abundant small oil droplets which become progressively less obvious in the cells nearer the centre of the organ. Starch is absent from the epidermal cells, but small starch grains are present in the sub-epidermal cells, and at the 40-day stage large starch grains are abundant in the central aerenchymatous tissue. At this stage there is therefore microchemical evidence of gradients for both fat and starch concentration, operating in opposite directions across the haustorium. In the closely related coconut palm the morphological changes during germination are similar to those in the oil palm, the endosperm being absorbed by a cotyledonary haustorium. In a microchemical study, Kirkwood and Gies (1902) found a pattern of starch and oil distribution in the coconut haustorium similar to that just described for the oil palm, oil being abundant in the epidermal cells whereas starch was present in all cells except those of the epidermis.

As absorption of the oil palm endosperm ceases, the epidermal cells of the haustorium undergo changes and starch grains appear in them; starch grains in the internal cells at this stage show signs of erosion (a similar effect was noted by Yampolsky, 1922). Later stages than this could not be examined owing to complete bacterial breakdown of the haustorium soon after 160 days.

No accumulation of oil was detected in either the root or shoot at any developmental stage and very little starch occurred in the root. As the seedling enlarges the base of the shoot becomes swollen and packed with starch, smaller quantities being detectable in the expanded leaves.

TABLE I

General components of the whole plant and of 'endosperm'

(g./50 seedlings*)

	Days after planting out					
	Ungermd.	0	20	40	90	160
WHOLE PLANT						
Dry wt.	40·36	34·56	32·67	37·11	48·62	88·24
	40·71	37·75	33·06	36·42	47·02	77·33
Lipid extract	20·06	18·39	15·38	14·88	5·56	1·79
	22·88	19·49	16·45	13·97	5·34	1·60
Carbohydrate (starch, sucrose and reducing sugars)	0·38	0·36	0·71	—	—	5·32
	0·37	0·34	0·67	—	—	4·20
Residual dry wt. (dry wt. minus determined constituents)	19·92	15·81	16·58	—	—	81·13
	17·46	17·92	15·94	—	—	71·53
'ENDOSPERM'						
Dry wt.	40·36	33·70	27·58	21·40	9·02	2·66
	40·71	36·75	28·13	21·01	8·86	2·49
Lipid extract	20·06	18·30	14·67	13·04	3·74	0·43
	22·88	19·37	15·65	12·34	3·77	0·36
Starch	0·168	0·064	0·039	0·035	0·022	0·009
	0·157	0·063	0·037	0·028	0·020	0·008
Sucrose	0·205	0·135	0·075	0·037	0·050	0·007
	0·207	0·108	0·066	0·030	0·043	0·004
Reducing sugars	0·006	0·033	0·017	0·012	0·019	0·005
	0·006	0·032	0·013	0·016	0·013	0·008
Residual dry wt.	19·92	15·17	12·78	8·28	5·19	2·21
	17·46	17·18	12·36	8·60	5·01	2·11
Non-saponifiable material	0·38	0·08	0·06	0·03	0·03	0·01
	0·35	0·07	0·07	0·02	0·02	0·01
Total fatty acids	18·00	15·28	13·30	9·93	3·29	0·29
	18·72	14·91	11·43	8·44	3·14	0·29
Combined fatty acids (chromatographic)	18·06	12·95	8·54	2·29	0·99	0·04
	(18·88)†	(13·61)	(9.69)	(2·63)	(2·12)	(0·07)
Free fatty acids (chromatographic)	0·66	1·96	2·89	6·15	2·15	0·25
	(4)‡	(13)	(25)	(73)	(69)	(86)

* The figures refer to 2 duplicate samples, the top figure for every component representing the same sample. Figures for combined fatty acids and free fatty acids refer to the 2nd sample.

† Extracted wt. in g./50 seedlings before chromatography.

‡ Percentage of total fatty acids.

2. *Analytical results. General.* Lipids account for about half the dry weight of the oil palm seed and there is very little reserve carbohydrate in the form of starch, sucrose, or reducing sugars (about 1 per cent.). The remainder of the fat-free dry weight contains protein (about 10 per cent.), 'fibre' (about 7 per cent.) and unidentified nitrogen-free material (Winton and Winton, 1932). As germination proceeds these endosperm reserves are slowly depleted causing a normal initial fall in dry weight, until photosynthesis commences as the shoot appears above ground (in this experiment at about 20 days). In the older seedling stages, carbohydrate (starch, sucrose, and reducing sugars) appears in increasing quantity while the weight of lipid falls to a low value (about 2–3 per cent. of the dry weight) as the reserve fat finally disappears (Table I).

'*Endosperm*'.[1] General analytical results appear in Table I which illustrates the gradual decrease in all 'endosperm' constituents as germination proceeds. By the 160-day stage there is a total loss of 94 per cent. of the original dry weight, 98 per cent. of the lipid, over 99 per cent. of carbohydrate (starch, sucrose, and reducing sugars) and 88 per cent. of 'residual dry weight' (dry weight minus determined constituents). The tissue remaining at 160 days consists almost entirely of thick-walled testa (see Morphology and Anatomy), and the evidence suggests that the entire true endosperm is absorbed or respired.

Amounts of the three determined carbohydrates are always low; starch shows a general fall over the whole period, as do reducing sugars after an initial slight rise. Sucrose decreases at first but then shows a slight rise between 40 and 90 days, which could be accounted for either by starch loss or by loss of 'residual dry weight' over the same period. These consistently low levels of carbohydrate suggest that fat is not converted to carbohydrate within the 'endosperm', unless this process is accompanied by extremely rapid carbohydrate transfer or metabolism.

In the lipid fraction, the amount of non-saponifiable material is low, showing little variation from 0 to 160 days. Total fatty acid falls regularly until 90 days have elapsed and then rather more slowly, closely paralleling lipid extract. Combined fatty acids behave somewhat similarly, although decreasing more rapidly until 40 days have passed, and then more slowly. The relation between free and combined fatty acids is of interest. Free fatty acids are at a low level in the ungerminated seed, but a steady rise then occurs until the 40-day stage, corresponding to a rapid decrease in combined fatty acids. From 40 to 160 days there is a decline in the free-fatty-acid content per seed, but as can be seen from Table I the *proportion* of free acid continues to climb reaching 86 per cent. in the final stage. There is close agreement throughout between acidity of the lipid extract, and acidity of sodium carbonate-extracted free fatty acids.

Analyses of the oil from ungerminated *Elaeis guineensis* seeds have already been published (Crombie and Boatman, 1955) and the present results (Table II) are substantially similar. Lauric acid (C_{12}) is the major constituent, and

[1] Inverted commas are used since in every case the endosperm sample included the testa.

TABLE II

Composition of the 'endosperm' combined fatty acids and free fatty acids

Mg./50 seedlings. Normal chromatographic method percentage in round brackets; square brackets, U=UV determined percentage, H=hydrogenation determined

Stage	Saturated acids C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{24}	Unsaturated acids Oleic	Linoleic	Iodine value (brackets = calculated from chrom. results)	Volatile acids (mg. equiv.)
COMBINED FATTY ACIDS												
Ungerm.	505 (2·8)	543 (3·0)	8,016 (44·4)	3,031 (16·8)	1,892 (10·5)	697 (3·8)	75 (0·4)	66 (0·4)	2,849 (15·8)	387 (2·1) [2·3U]	18·3 (18·0)	4·9
0 .	206 (1·6)	272 (2·1)	6,205 (47·9)	2,270 (17·5) [17·6H]	1,318 (10·2) [9·8H]	384 (3·0)	71 (0·5)	15 (0·1)	2,084 (16·1) [16·5H]	129 (1·0) [0·9H]	16·8 (16·3)	2·9
20 .	104 (1·2)	155 (1·8)	3,740 (43·8)	1,473 (17·2)	897 (10·5)	239 (2·9)	16 (0·2)	7 (0·1)	1,694 (19·8)	216 (2·5)	21·0 (22·3)	1·6
40 .	61 (2·7)	60 (2·6)	897 (39·2)	364 (15·9)	264 (11·5)	60 (2·6)	3 (0·1)	3 (0·1)	508 (27·2)	70 (3·1)	30·9 (30·1)	0·5
90 .	2 (0·3)	8 (0·9)	330 (33·4)	146 (14·8) [15·4H]	134 (13·5) [11·8H]	30 (3·0)	3 (0·3)	4 (0·4)	280 (28·3) [30·1H]	51 (5·1) [4·9H] [5·0U]	33·3 (34·7)	—
160 .	0·5 (1·3)	0·3 (0·7)	8·0 (20·3)	2·7 (6·9)	4·9 (12·4)	0·6 (1·6)	0·1 (0·2)	0·3 (0·7)	17·9 (45·4)	4·2 (10·5)	58·8 (59·8)	—
FREE FATTY ACIDS												
Ungerm.	9 (1·4)	17 (2·6)	302 (45·5)	123 (18·5)	92 (13·8)	26 (3·9)	3 (0·5)	7 (1·1)	76 (11·5)	8 (1·2)	13·3 (12·5)	—
0 .	182 (9·2)	76 (3·9)	875 (44·7)	324 (16·6)	151 (7·7)	71 (3·6)	9 (0·5)	4 (0·2)	234 (12·0)	31 (1·6)	13·7 (14·5)	—
20 .	53 (1·8)	72 (2·5)	1,392 (48·1)	482 (16·7)	326 (11·3)	128 (4·4)	13 (0·4)	7 (0·2)	370 (12·9)	50 (1·7)	16·1 (14·7)	—
40 .	138 (2·2)	165 (2·7)	2,566 (41·7)	1,078 (17·5) [17·1H]	723 (11·8) [10·6H]	205 (3·3)	25 (0·4)	22 (0·4)	1,130 (18·4) [19·7H]	98 (1·6) [2·1H]	20·8 (19·4)	—
90 .	23 (1·1)	36 (1·7)	757 (35·3)	328 (15·7)	297 (13·8)	80 (3·7)	6 (0·3)	7 (0·3)	537 (25·0)	67 (3·1) [3·7U]	30·2 (28·1)	—
160 .	0·7 (0·3)	3·4 (1·4)	61·9 (24·9)	21·0 (8·4)	29·7 (11·9)	8·8 (3·5)	0·9 (0·4)	3·3 (1·3)	96·9 (38·9)	22·4 (9·0)	52·9 (51·3)	—

saturated fatty acids varying in chain length from C_8 to C_{20} or more constitute about 82 per cent. of the total. Small amounts of long-chain saturated acidic material eluting slowly in 90 per cent. acetone are calculated as C_{24}, but may represent more than one acid. Oleic (approximately 16 per cent.) is the major unsaturated acid accompanied by about 2 per cent. of linoleic acid. An octadecatrienoic acid (non-conjugated), not previously reported in palm kernel oil, was estimated, by UV absorption after isomerisation ($\lambda_{max} = 268m\mu$), to constitute about 0·2 per cent. of the total fatty acids, and no conjugated diene or triene acids were detected (Boatman, 1956). Scrutiny of the hydrogenation and oxidation figures, which are available in a number of cases, indicates that it is improbable that unsaturated acids of chain length other than C_{18} are present in significant amounts.

During germination the weight per seed of every acid in the combined acid fraction falls (the slight increase in linoleic acid between 0 and 20 days is within the experimental error). The percentage composition of this same fraction shows, however, a considerable change. The proportions of lauric, myristic, palmitic, and stearic acids fall, and the proportions of oleic and linoleic acids rise. These changes are also reflected in the rising iodine values (Table II). Volatile combined fatty acids were determined directly by steam distillation but were always low in amount, the values agreeing well with the chromatographic results for acids of chain length C_{10} or less.

The composition of the free fatty acids is also shown in Table II and weight increases (per seed) of each acid occur until the 40-day stage, after which time there is a general fall. The sharp increase in weight of C_8 acid at 0 days seems anomalous and may be due to transport at this stage. There is a tendency throughout for oleic and possibly linoleic to be at a lower proportionate concentration in the free acids than in the combined. Apart from this, changes in composition of the free fatty acids are remarkably similar to those in the combined acid fraction, showing a steady rise in unsaturation as germination proceeds.

Volatile free fatty acids were determined by steam distilling a separate sample of seedlings, with the results shown in Table III. Although the figures are not strictly comparable with those of the main experiment, there is no evidence to suggest that short-chain volatile free fatty acids accumulate during germination.

The remarkable rise in acidity of the lipid fraction during its utilization suggested the activity of a lipase and further evidence is provided by the rise in hydroxyl value from 6·84 in the ungerminated seed to 14·65 in the partly digested 'endosperm' (Hardman, unpublished). If a lipase, accompanied by cell-wall digesting enzymes, were secreted by the outer layers of the haustorium, the gradual disintegration of the 'endosperm' with liberation of free fatty acids would be explained. Alternatively the enzyme may be present in the 'endosperm', being activated by cellular disruption, or by a substance secreted by the haustorium. In an attempt to solve this problem, a number of experiments were performed to test for lipase activity in concentrated extracts from

(1) total 'endosperm', (2) 'soft' endosperm surrounding the haustorium, and (3) separated haustoria. Different substrates (palm kernel oil, palm oil, and Tween 80)[1] were tested and acetic acid was employed as an activator in some experiments. In no case could lipase activity be demonstrated by increase in lipid acidity following 24 hours' incubation at 30° C., although the same technique was effective in demonstrating the presence of lipase in *Ricinus*,

TABLE III

'Endosperm' and haustorium—volatile free fatty acids

(Mg. equiv./50 seedlings)

Days after planting out*	'Endosperm'	Haustorium
10	0·16	0·01
	0·20	0·02
40	0·30	0·08
	0·40	0·10
90	0·25	0·18
	0·18	0·14

* These stages refer to seedlings grown on a different occasion from the main experiment.

Citrullus, *Helianthus*, and *Rapa* (Hardman, unpublished). It is therefore assumed that the enzyme has low activity and cannot be demonstrated in short-term experiments. Similar findings have been reported in the coconut (Kirkwood and Gies, 1902) where germination, as in the oil palm, is a protracted process.

Since these experiments were unsuccessful, an attempt was made to determine whether lipase activity was greater in the zone adjacent to the haustorium,

TABLE IV

Composition of the free and combined acid fractions in the 'soft' and 'hard' endosperm

(Wt. percentage of fatty acid)

	'Soft' endosperm		'Hard' endosperm	
Fatty acid	Free acids	Combined acids	Free acids	Combined acids
SATURATED				
C_8 or lower	0·3	5·5	0·3	3·2
C_{10}	0·3	4·7	0·3	3·3
C_{12}	22·1	52·5	25·4	47·1
C_{14}	34·5	13·4	28·6	17·5
C_{16}	17·9	6·2	19·1	9·4
C_{18}	8·3	3·4	6·8	2·7
C_{20}	trace	0·3	0·3	trace
C_{24}	0·6	0·4	0·6	0·5
UNSATURATED				
Oleic	15·2	13·2	16·8	15·0
Linoleic	0·8	0·4	1·8	1·3
Total weight (g.)	0·185	1·843	0·113	10·778

[1] Polyoxethylene sorbitan mono-oleate (ex. Atlas Powder Co.).

by separating 'soft' endosperm from the remaining 'hard' endosperm and estimating the amounts of free and combined acids in each fraction (Table IV). There is a much higher proportion of free acid (approx. 10 per cent.) in the 'soft' endosperm than in the 'hard' endosperm (approx. 1 per cent.), indicating greater lipase activity in the former region.

An interesting point arises from comparison of the composition of the free and combined fatty acids. In the main experiment these were of very similar composition throughout germination, but when the second experiment (Table IV) was undertaken it was found that the free acids were richer in C_{14} to C_{24} and poorer in C_8 to C_{12} chain lengths, relative to the combined acids. An important difference between the two experiments is that the seedlings in the second experiment grew more slowly, their free-acid content was much lower

TABLE V

General components of the haustorium

(g./50 seedlings)

Each figure is the mean of 2 duplicate samples, which agree closely (see Table I)

	Days after planting out				
	0*	20	40	90	160
Dry wt.	0·421	1·947	4·067	4·241	3·113
Lipid extract (Brackets % of dry wt.)	0·087 (21)	0·695 (36)	1·545 (38)	1·111 (26)	0·420 (14)
Starch	0·030	0·128	0·236	0·244	0·081
Sucrose	0·067	0·288	0·533	0·590	0·274
Reducing sugars	0·004	0·043	0·150	0·356	0·588
Residual dry wt.	0·233	0·795	1·604	1·940	1·751
Non-saponifiable	0·004	0·006	0·018	0·008	0·008
Total fatty acids (chromatographic)	0·053	0·467	1·257	0·790	0·371
Combined fatty acids (chromatographic)	0·052 (0·061)†	0·464 (0·610)	1·251 (1·354)	0·788 (0·914)	0·368 (0·379)
Free fatty acids (Extracted wt.)	0·002 (2·8)†	0·004 (0·8)	0·006 (0·5)	0·002 (0·2)	0·003 (0·7)

* Ungerminated embryo: dry wt. = 0·0705, lipid extract = 0·019 (27% of dry wt.)
† Significance as in Table I.

(2·4 compared with 25 per cent.), and the 'endosperm' was digested more slowly. Under these conditions hydrolysis seems less complete. It may be then, that when reaction proceeds only to a small extent, selectivity factors operate which are masked when hydrolysis has proceeded to a much larger extent.

Haustorium. Changes in components of the haustorium during germination are shown in Table V. There is an increase in dry weight until 90 days followed by a fall. The organ reaches nearly full size by 90 days and after this increases only slightly in volume. After 160 days in the present experiment the haustorium rots away and no further analyses were made. The decrease in dry weight

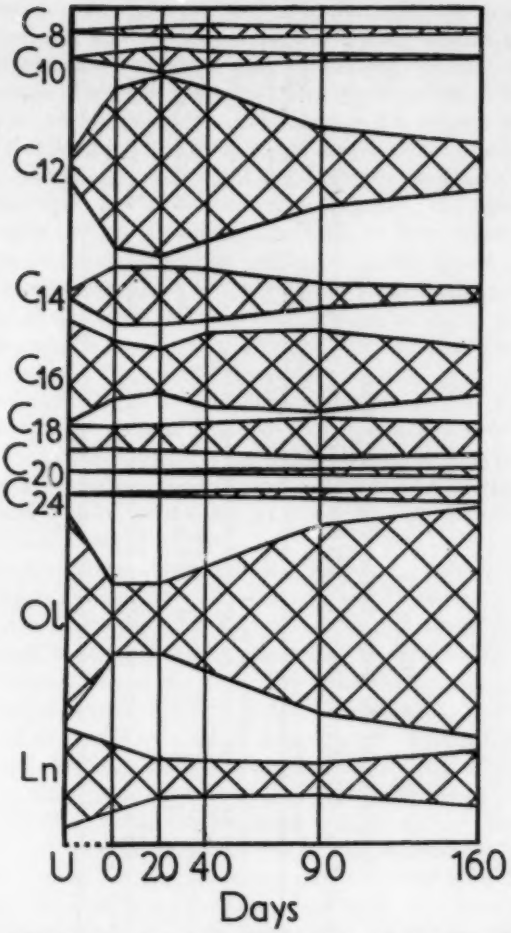


FIG. 2. Weight percentage composition of the haustorium fatty acids at different stages of germination. C_8—C_{24}, saturated fatty acids of the appropriate chain length; Ol, oleic acid; Ln, linoleic acid. A dotted line is used between the ungerminated stage (U) and 0 days to indicate that the time interval is unknown.

after 90 days is due to a fall in food reserves (lipid and total carbohydrate), the residual dry weight showing little change. The weight of lipid extract per haustorium increases until 40 days, remains approximately constant from 40 to 90 days, and then falls; so does the percentage of lipid present, which is, however, always lower than in the endosperm. These changes may be related to the gradual decrease in surface/volume ratio as the organ reaches full size, causing absorption to become slower than breakdown within the haustorium.

Table VI

Composition of the haustorium combined fatty acids

(Mg./50 seedlings: other conventions as in Table II. Each figure is the mean of duplicate samples)

Stage	Saturated acids								Unsaturated acids		Iodine value	Volatile acids (mg. equiv.)
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{24}	Oleic	Linoleic*		
Embryo .	—	—	0·5 (3·8)	0·1 (0·7)	3·0 (22·9)	0·7 (5·4)	—	—	6·0 (45·6)	2·9 (21·6)	—	—
0 days .	1·2 (2·3)	1·4 (2·7)	17·9 (34·8)	6·1 (11·0) [12·7H]	6·8 (13·2) [13·6H]	2·3 (4·5)	0·2 (0·4)	0·2 (0·4)	8·1 (15·8) [15·0H]	7·2 (14·0) [12·6H]	40·1 (39·6)	—
20 ,,	13·5 (2·9)	24·6 (5·3)	188·0 (40·6)	56·2 (12·2)	47·5 (10·3)	26·1 (5·6)	2·0 (0·4)	1·4 (0·3)	65·7 (14·2)	37·9 (8·2)	29·0 (27·8)	0·12
40 ,, .	41·4 (3·3)	30·9 (2·5)	423·7 (33·8)	121·0 (9·7) [10·1H]	199·0 (15·9) [14·8H]	70·1 (5·6)	5·0 (0·4)	8·1 (0·6)	262·7 (21·0) [21·1H]	88·8 (7·1) [6·7H] [6·4U]	34·1 (31·7)	0·34
90 ,, .	6·4 (0·8)	7·4 (0·9)	129·5 (16·4)	29·8 (3·8)	149·2 (18·9)	70·0 (8·9)	10·0 (1·3)	17·7 (2·2)	319·4 (40·5)	49·0 (6·2)	48·8 (47·8)	0·05
160 ,, .	1·7 (0·5)	1·9 (0·5)	35·8 (9·7)	11·7 (3·2)	38·7 (10·5)	26·0 (7·1)	6·8 (1·8)	7·6 (2·1)	190·7 (51·8)	47·5 (12·9)	70·3 (69·8)	0·02

* Ultraviolet absorption methods could not detect any conjugated diene or triene acids, or unconjugated triene acid.

Between 40 and 90 days there is a change in the main reserve material from fat to carbohydrate, starch, sucrose, and reducing sugars accumulating. Sucrose and starch show a rise until 90 days followed by a fall, whereas reducing sugars continue to increase throughout the whole period. The distribution of starch grains and fat globules within the cells has already been recorded (Morphology and Anatomy), and suggested that some fat was converted into carbohydrate. No definite conclusions can, however, be drawn from the evidence available, since the haustorium carbohydrate could either have been translocated from the photosynthesizing shoot, or have arisen from carbohydrates in the 'residual dry weight' fraction of the endosperm.

The chief component of the lipid fraction is combined fatty acids, the weight changes of which are closely similar to those of the lipid extract; non-saponifiable material is low in weight throughout and shows no noteworthy change. The most interesting feature is the consistently low proportion of free fatty acids, both volatile (Table III) and longer-chain (Table V), particularly in view of the high concentration of free fatty acid in the endosperm immediately around the haustorium. This indicates that if free fatty acids are absorbed they are rapidly re-esterified, and invites comparison with similar processes believed to occur during fat absorption in the animal intestine (e.g. Reiser, 1954; Borgström, 1951).

Detailed analyses of the combined acid fraction are seen in Table VI, included in which is an analysis of the ungerminated embryo. The embryo is of course not strictly analogous to haustorium since it also contains root and shoot initials, but it is estimated by inspection, that about three-quarters of the embryo represents future haustorium (see also Henry, 1951). This embryo fat is seen to have an entirely different constitution from that of the surrounding endosperm, but as germination begins the amount of fat in the haustorium rises rapidly and its composition comes to resemble that of the endosperm (Fig. 2). After 40 days when the weight of lipid is falling the constitution alters again until at 160 days, when the fat content is relatively low ($\sim$ 14 per cent.), the composition once again resembles that of the ungerminated embryo, and may represent a 'protoplasmic' fat characteristic of this tissue. Such changes strongly suggest that between 0 and 40 days lipids are absorbed from the endosperm by the outer layers of the haustorium. Glycerides (no attempt was made to differentiate between mono-, di-, and triglycerides) and free acids are present in the 'soft' endosperm around the haustorium and are theoretically available for absorption. Free fatty acids are presumably absorbed, either in the free state or after re-esterification, since the quantity in the endosperm eventually falls.

Some interesting differences in the rates of accumulation and disappearance of fatty acids within the haustorium are found (Fig. 3, Table VI). The C_8, C_{10}, C_{12}, and C_{14} saturated acids, and also linoleic acid, show a rapid rise between 0 and 40 days, followed by a rapid fall between 40 and 90 days and a slower fall until 160 days. The amounts of volatile combined acids were again found to be very low, corresponding approximately to the weights of C_8 and

C_{10} acids as in the endosperm samples. The C_{16} and C_{18} saturated acids behave rather similarly to the shorter-chain acids, although there is a less marked decline between 40 and 90 days and a faster decline later. Longer-chain saturated acids (C_{20}, and an acid eluting slowly denoted as C_{24}), present in relatively small amounts, increase until 90 days and then decrease. This behaviour is seen even more clearly in the case of oleic acid which forms a relatively large proportion of the total fatty acid. These results will be considered in greater detail in the discussion.

Shoot. Changes in the main components of the developing shoot are shown in Table VII (the value at 0 days is that for unseparated root and shoot, both

TABLE VII

General components of the shoot and root

(g./50 seedlings. Each figure is the mean of duplicates)

	Days after planting out					
	0*	20	40	90	160	350
SHOOT						
Dry wt.	0·511	1·678	7·678	23·261	51·890	110·018
Lipid Extract (Brackets = % of dry wt.)	0·018 (3)	0·039 (2)	0·157 (2)	0·496 (2)	0·747 (1)	1·794 (2)
Starch	0·008	0·015	0·072	—	0·923	—
Sucrose	0·001	0·024	0·035	—	0·287	—
Reducing sugars	0·023	0·036	0·275	—	1·740	—
Non-saponifiable	0·004	0·001	0·005	—	0·038	0·315
Combined fatty acids	0·0041 (0·0075)†	0·0180 (0·0262)	0·0688 (0·1027)	—	0·2587 (0·5885)	0·7562 (1·2664)
Free fatty acids (Extracted wt.)	0·0001 (2·5)†	0·0004 (2·2)	0·0008 (1·1)	—	0·0019 (0·8)	0·0024 (0·3)
ROOT						
Dry wt.)	—	1·392	3·815	11·374	25·112	56·745
Lipid extract (Brackets = % of dry wt.)	— —	0·017 (1)	0·030 (1)	0·089 (1)	0·132 (0·5)	0·243 (0·5)
Starch	—	0·005	—	—	—	0·058
Sucrose	—	0·001	—	—	—	0·033
Reducing sugars	—	0·026	—	—	—	0·759
Non-saponifiable	—	0·003	—	—	—	0·088
Combined fatty acids	— —	0·002 (0·004)†	—	—	—	0·034 (0·082)†
Free fatty acids	— —	0·002 (5·0)†	—	—	—	0·001 (2·9)†

* Combined root and shoot.
† Significance as in Table I.

of which are small at this stage). Dry weight increases exponentially over the period studied and there is little change in proportion of the components, by far the largest fraction (over 90 per cent.) being 'residual dry weight'. The lipid content is low (about 2 per cent.) and starch, sucrose, and reducing sugars

together account for about 5 per cent. of the dry weight, reducing sugars forming the largest fraction of the total. Only about half the lipid extract can be accounted for as non-saponifiable material or fatty acid and except for the 0-day samples (23 per cent. total fatty acid), the proportion of total fatty acid remains fairly constant (from 37 to 49 per cent.). Non-saponifiable material forms about 23 per cent. of the 0-day sample, 3 to 6 per cent. in the 20-, 40-, and 160-day samples, and 18 per cent. of the lipid extract at 350 days. Free fatty acids remain at a very low level throughout.

Detailed analyses of the combined fatty-acid fraction are recorded in Table VIII. All fatty acids present in the endosperm occur also in the shoot but in very different proportions, and there is in addition an unconjugated octadecatrienoic acid, probably linolenic acid. The fat from the shoot at 350 days is highly unsaturated.

Linolenic ($\sim$ 33 per cent.) and linoleic ($\sim$ 22 per cent.) are the major unsaturated acids and their presence was confirmed by U.V. absorption after alkaline isomerization, and hydrogenation to stearic acid. In addition small amounts of conjugated diene ($\lambda_{max} = 233$ mμ) and triene ($\lambda_{max} = 268$ mμ; subsidiary maxima at 258 and 278 mμ) acids were detected by their U.V. absorption and estimated (in the 160-day sample) to form not more than 3·8 and 0·3 per cent. respectively of the total fatty acids. No evidence for conjugated or unconjugated tetraene material was obtained at any stage. Conjugated diene, triene and, in addition, tetraene acids have been recorded in certain other green-leaf fatty acids (Zirm *et al.*, 1955). In view of the close agreement between oxidation and hydrogenation figures, it seems unlikely that there is any significant amount of unsaturated material of chain length other than C_{18}.

The major saturated acid is palmitic (about 18 per cent.) accompanied by minor amounts of C_8, C_{10}, C_{12}, C_{14}, C_{18}, C_{20}, and C_{24} acids (the last acid was not studied in any detail and may represent a mixture).

In the 0-day stage the composition is very different from that later (the major acids are palmitic and linoleic). The fatty-acid composition does in fact simulate that for later stages of the root (Table VIII). During shoot development most of the saturated acids (C_8, C_{10}, C_{18}, C_{20}, C_{24}) show a steady increase in amount, with relatively small fluctuations in percentage. Lauric acid (C_{12}) shows a rather small decrease in amount at 40 days, which is reflected in a relatively large (and duplicated) increase in percentage at 20 days (12 compared with 1–4 per cent. at other stages). Myristic acid (C_{14}) also increases in percentage at this stage, but there is no decrease in its weight per seedling. The presence in the shoot of these acids, abundant in the endosperm and haustorium, raises the question of fatty acid transport, but evidence for it is slight. A more attractive possibility is that these acids are more important constituents of hypocotyl or unexpanded leaf fat than of expanded leaf fat (in this 20-day stage most of the leaves present are sheath leaves, whereas in later stages almost the whole shoot consists of expanded leaves). Palmitic acid (C_{16}) shows a continuous rise in amount, increasing in percentage from about

TABLE VIII

Composition of the shoot and root combined fatty acids

(Mg./50 seedlings, conventions as in Table II)

	Saturated acids								Unsaturated acids			
Stage	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{24}	Oleic	Linoleic	Linolenic	Longer-chain unsaturated
0* days .	0·10 (2·5)	0·01 (0·3)	0·06 (1·5)	0·15 (3·6)	1·16 (28·6)	0·12 (3·0)	0·03 (0·7)	0·22 (5·3)	0·29 (7·2)	1·79 (44·4)	0·12 (2·9)	0·00 (0·0)
SHOOT												
20 days†	0·5 (2·7)	0·2 (1·1)	2·2 (12·0)	1·2 (6·6)	5·4 (29·5)	1·1 (6·0)	0·2 (1·1)	0·4 (2·2)	0·2 (1·1)	4·8 (26·2)	1·3 (7·1)	0·8 (4·4)
40 „ †	2·0 (2·9)	0·5 (0·7)	1·0 (1·4)	1·8 (2·6)	26·5 (38·4)	3·0 (4·3)	1·1 (1·6)	3·6 (5·2)	0·8 (1·2)	16·1 (23·3) [22·3U]	12·5 (18·1) [15·4U]	0·2 (0·3)
160 „	5·1 (2·0)	1·5 (0·6)	3·1 (1·2)	8·3 (3·2)	74·5 (28·8)	19·4 (7·5)	4·1 (1·6)	13·0 (5·0)	27·1 (10·4)	41·0 (15·8) [15·7U]	60·5 (23·4) [25·4U]	1·3 (0·5)
350 „	26 (3·5)	5 (0·6)	30 (4·0)	23 (3·0) [3·4H]	134 (17·8) [19·2H]	35 (4·6)	5 (0·7)	4 (0·6)	60 (7·9) [6·3H]	169 (22·4) [22 oH] [21·8U]	252 (33·3) [30·7H] [35·4U]	12 (1·6)
ROOT												
20 days .	—	0·10 (4·9)	0·04 (1·9)	0·04 (2·2)	0·52 (26·9)	0·10 (5·3)	0·05 (2·4)	0·18 (9·1)	0·21 (10·9)	0·66 (33·9)	0·00 (0·0)	0·05 (2·5)
350 „ .	—	0·83 (2·5)	0·17 (0·5)	0·24 (0·7) [0·8H]	10·74 (31·8) [30·0H]	1·42 (4·2)	0·14 (0·4) [0·5H]	0·52 (1·6)	4·06 (12·0) [14·1H]	14·87 (44·0) [42·9H]	0·00 (0·0)	0·78 (2·3) [2·8H]

* Root and shoot analysed together at the 0-day stage. † Mean values from duplicate samples.

30 at 0 days to 38 at 40 days, then declining to 18 per cent. at 350 days.

The most striking changes occur in the unsaturated acid fraction; oleic acid shows a slight decrease at 20 days, followed thereafter by a continuous rise until 350 days. Its percentage is comparatively high at first, probably because of the presence of root tissue at 0 days. At the 20- and 40-day stages it drops to a low level, then rises to a higher value in the later stages. Linoleic acid increases in amount per shoot throughout seedling development, decreasing from an initial 44 per cent. at 0 days (almost certainly caused by the preponderance of root tissue which is characterized by a high linoleic acid content) to a more or less steady value. There is little linolenic acid present in the early stages but from 40 days onwards, as the leaves expand and turn green, both the proportion and total quantity rise. A similar production of this acid was demonstrated in expanding *Citrullus* cotyledons (Crombie and Comber, 1956), but was complicated in that tissue by concomitant disappearance of storage fat.

Root. Changes in weight of the major root constituents are shown in Table VII. As in the shoot, dry weight increases exponentially during germination, whereas the proportions of components remain relatively constant (lipid extract ~ 1, 'carbohydrate' ~ 3–4, 'residual dry weight' ~ 95–97 per cent.). Reducing sugars form the greatest part of the 'carbohydrate', both starch and sucrose being at very low concentrations throughout. In the lipid extract, which is very low in total amount, fatty acids and non-saponifiable material represent only about half the total weight—a similar situation to that in the shoot. The weight of 'free fatty acid' is low at 0 days (combined root and shoot), and at 350 days, but accounts for 55 per cent. of the total fatty acid at 20 days. However, as the *acidity* of this particular free acid extract is low, it seems probable that extraneous material is responsible for the high weight recorded.

Only two later stages of roots were examined for component fatty acids and these show saturated acids (C_{10} to C_{24}), oleic, linoleic, and longer-chain unsaturated acids, all increasing in total weight from 20 to 350 days (Table VIII). Linolenic acid is absent and no conjugated diene or triene acids were found. The fat composition is therefore totally different from that of the shoot.

The constitution of root fat changes remarkably little during development; linoleic and palmitic acids predominate and there is a smaller proportion (~ 11 per cent.) of oleic acid. As mentioned in connexion with the shoot, the composition of fat from combined root and shoot in the 0-day stage seems to correspond more closely to that of the root. Neither root nor shoot has a high fat content and it seems that each contains a low proportion of its own characteristic 'protoplasmic' fat.

DISCUSSION

As the 'endosperm' is slowly invaded by the developing haustorium both lipid and non-lipid material disappears from it. Only relatively low amounts

of lipid occur in the young seedling whose dry weight is composed almost entirely of non-lipid materials. Table IX shows the distribution within the different parts of the young plant of these compounds (compared on a carbon basis), and it is at once apparent that as in other germinating seeds, a major part of the original seed reserve material is lost by respiration (86 per cent. up to the 0-day stage, 60 per cent. between 0 and 20 days). Since the seedlings were grown in the light such calculations cannot be made after the shoot appeared above ground at about 20 days.

TABLE IX

Carbon balance sheet for early stages of germination before the start of photosynthesis

(g. C/50 seedlings)

Figures in brackets show total C lost over the period as percentage of C lost from the endosperm in the same period.

Component	Endosperm	Haustorium	Root+Shoot	Total	Period
Lipid . . .	−1·97	+0·05	+0·01	−1·91	Ungermd. → 0 days
Fat-free residue (as carbohydrate) .	−1·07	+0·08	+0·29	−0·70	
Total	−3·04	+0·13	+0·30	−2·61 (−86%)	
Lipid . . .	−2·78	+0·46	+0·03	−2·29	0 → 20 days
Estimated carbohydrates . .	−0·04	+0·15	+0·03	+0·14	
Residual dry weight (as carbohydrate) .	−1·07	+0·17	+0·72	−0·18	
Total	−3·89	+0·78	+0·78	−2·33 (−60%)	

It was of particular interest to determine the form in which the 'endosperm' reserves were absorbed by the haustorium. Translocation of fat has previously been suggested in other germinating fatty seeds, but the evidence has been questioned by Rhine (1926) who demonstrated prior conversion into carbohydrate. In *Ricinus* considerable amounts of sugar accumulate in the endosperm (*inter al.* Houget, 1942, 1943; Desveaux and Kogane-Charles, 1952; Yamada, 1955), which has a very low respiratory quotient characteristic for fat into carbohydrate conversion (Murlin *et al.* 1933; Yamada, 1955). In *Elaeis*, however, sugars do not accumulate in the 'endosperm' at any stage, and measurement of the respiratory quotient of this tissue (Hardman, unpublished) gave values of approximately 0·8 indicating again no active conversion of fat into carbohydrate.

Instead there is strong evidence suggesting that lipids are absorbed by the haustorium. While fat is disappearing rapidly from the 'endosperm', lipids of the 'endosperm' type, not originally present in the embryo, begin to accumulate in the haustorium. This finding and the microchemical observation of a gradient in fat concentration from the outer to inner layers strongly

suggests that lipids are absorbed by the haustorium without prior conversion into carbohydrate.

The morphology of *Elaeis* is very different from that of fatty seedlings previously investigated and the epidermal cells of the haustorium appear to be specialized for absorption. The whole process raises a comparison with fat absorption in the animal intestine. In the latter, as in the oil palm 'endosperm', lipid hydrolysis products, including free fatty acids, are in close contact with the absorbing tissue. During absorption from the animal intestine, free acids are re-esterified, and a similar process seems probable in *Elaeis*, since no free fatty acids were found at any time in the haustorium.

TABLE X

Comparison of a testa analysis (*Carsten* et al. *1945*) *with 90-day 'endosperm'*

	Testa	90-day 'Endosperm'
Dry wt. as % of ungerminated seed	20·3	22·1
% Fat in dry wt.	40·2	41·1
Iodine value of fat	31·1	30·6
Wt. % of Fatty Acid		
Saturated		
C_8 or lower	0·6	0·9
C_{10}	4·4	1·3
C_{12}	35·7	34·8
C_{14}	16·0	15·5
C_{16}	10·7	13·3
C_{18}	1·5	3·6
C_{20}	—	0·3
C_{24}	—	0·3
Unsaturated		
Hexadecenoic	0·1	—
Oleic	26·0	25·9
Linoleic	5·0	4·1

It seemed of special interest to determine whether component fatty acids disappeared at proportional rates or whether some degree of selectivity was apparent. Selectivity could be due to one of two main causes or to a combination of them: first, differential absorption of fatty acids by the haustorium, and, second, differential breakdown of the absorbed acids within the haustorium. The problem is thus more complex than in *Citrullus* (Crombie and Comber, 1956), where the cotyledonary fat is broken down *in situ*. The two main problems will now be discussed separately.

Absorption of fatty acids will obviously be considerably influenced by the composition of the fat surrounding the absorbing organ. At most stages fatty acids are available in both the free and combined forms (mono-, di-, and triglycerides are not distinguished); therefore if one of these fractions is more readily absorbed, uptake of individual acids will reflect the relative quantities in this preferred form. Such a complicating factor is unlikely to be important in the main experiment since at all stages free and combined acids show a generally similar fatty acid composition.

The composition of fat available for absorption by the haustorium will also be affected by any gradient in fatty acid composition from the inner to the outer tissues of the 'endosperm', since, as the haustorium enlarges the proportion of intact outer layers in each 'endosperm' sample becomes larger (Fig. 1). In the final stage (160 days) very few true endosperm cells remain and much of the lipid extract derives from the testa, whose cells possess an oil which stains deeply with osmium tetroxide. The striking increase in unsaturation of the remaining 'endosperm' oil during germination may therefore be interpreted as being due not to selective uptake of saturated acids, but to a progressive increase in the proportion of testa in successive samples.

In order to test this, a 'testa' sample (Carsten *et al.*, 1945), obtained by careful scraping away of the outer layers from Oil Palm kernels, was compared on a basis of dry weight, lipid extract, and fatty acid composition with a late stage (90 days) of 'endosperm' (Table X). There is a most convincing similarity, indicating that the above explanation is correct.

TABLE XI

Percentage composition of endosperm total fatty acids after 'testa' correction

(Values of the 90-day sample subtracted from each preceding stage)

Fatty acid		Ungermd.	Days after planting 0	20	40
Saturated	C_8	3·4	3·4	1·6	2·8
	C_{10}	3·5	2·8	2·2	3·2
	C_{12}	46·4	50·1	49·4	44·8
	C_{14}	17·0	17·9	17·6	18·1
	C_{16}	9·9	9·7	9·5	10·7
	C_{18}	3·7	3·2	3·1	3·1
	C_{20}	0·4	0·7	0·3	0·4
	C_{24}	0·4	0·1	0·0	0·2
Oleic		13·4	11·8	14·9	15·6
Linoleic		1·9	0·3	1·4	1·1

If this complication is removed (by subtracting the weights of each acid at the 90-day stage from those in each of the previous stages), the composition of the remaining endosperm fat remains remarkably constant during its slow disappearance (Table XI). Selective absorption of fatty acids does not therefore occur to any significant extent while over 75 per cent. of the fat is being used (21 to 3·7 g.). Since breakdown of endosperm cells must precede absorption of their contents by the haustorium, a likely explanation of this lack of selectivity in uptake is that the dissolution process is slower than absorption.

It is interesting to note that the changes in fatty acid composition of the 'endosperm' during fat disappearance are almost exactly the reverse of those which occur in the same tissue during fat formation (Crombie, 1956), especially since it was noted previously that the testa is fully developed at an early stage in kernel development (endosperm still semi-liquid), and fat-containing tissue develops centripetally.

Rather active breakdown of fatty acids probably occurs in the haustorium, since the total quantity of fat which it contains it considerably less than the amount which disappears from the 'endosperm'. This conclusion is also supported by the high losses due to respiration (see Table IX). It has already been shown that no appreciable selective absorption of fatty acids occurs, and the next problem was to consider whether there was any evidence indicating selective breakdown. It is noteworthy that in this tissue, as in *Citrullus*, no free acids accumulate during active fat breakdown.

Since the composition of the absorbed fat remains approximately constant until the 90th day, selective breakdown should be apparent from a study of the fatty acids accumulated by the haustorium. Fig. 3 shows clearly that there are differences in rates of accumulation and disappearance of lower saturated acids (C_8 to C_{14}), higher saturated acids (C_{16} to C_{24}) and unsaturated acids (oleic and linoleic), a fact of interest since different enzymes are believed to be concerned in the metabolism of fatty acids of different chain length and degree of unsaturation in animals (see Green, 1954) and in plants (Humphreys *et al.*, 1954; Stumpf, 1956).

Fat metabolism in the haustorium can be considered in two phases; first, that in which fat absorption is faster than fat breakdown (from the start of germination until 40 days), and second, that in which fat breakdown is faster than fat absorption (from 40 days until the end of the experiment). Since absorption, which has been shown to be relatively unselective, is faster in the first phase it will tend to mask any selectivity in the relatively slow fatty acid breakdown process, as determined from examination of the fat retained in the haustorium. In the second phase, however, when fatty acid breakdown is faster, selectivity should be immediately apparent. Scrutiny of the results (Figs. 2 and 3) shows quite clearly that in this second phase lower saturated acids (C_8 to C_{14}) are metabolized more rapidly than either long-chain saturated acids (C_{16} and above) or unsaturated acids. Some caution is necessary in interpreting results after the 90-day stage, since the composition of the absorbed fat may be affected by the testa. However, a similar effect occurs also in the first phase though, as is to be expected, it is less obvious. It is particularly interesting to note that acids uncharacteristic of the haustorium are broken down at a faster rate than those which predominate in its own fat (palmitic, stearic, oleic, and linoleic) at an early (embryo) and late (160 days) stage. A rather similar result was obtained by Longenecker, 1939, in an interesting experiment in which coconut oil (in composition this closely resembles palm kernel oil) was fed to fasted rats. Appreciable quantities of shorter-chain saturated acids accumulated in the depot fat and the relative rates of removal were studied during subsequent inanition. Lower molecular weight acids (e.g. C_{12}) were found to be removed relatively more rapidly, as in the present experiment with the oil palm. Longenecker pointed out that, although his results would support a preferential utilization of shorter-chain acids, other interpretations based on assumptions as to distribution of fatty acids within the glycerides, were possible. A similar complication may be operative in the

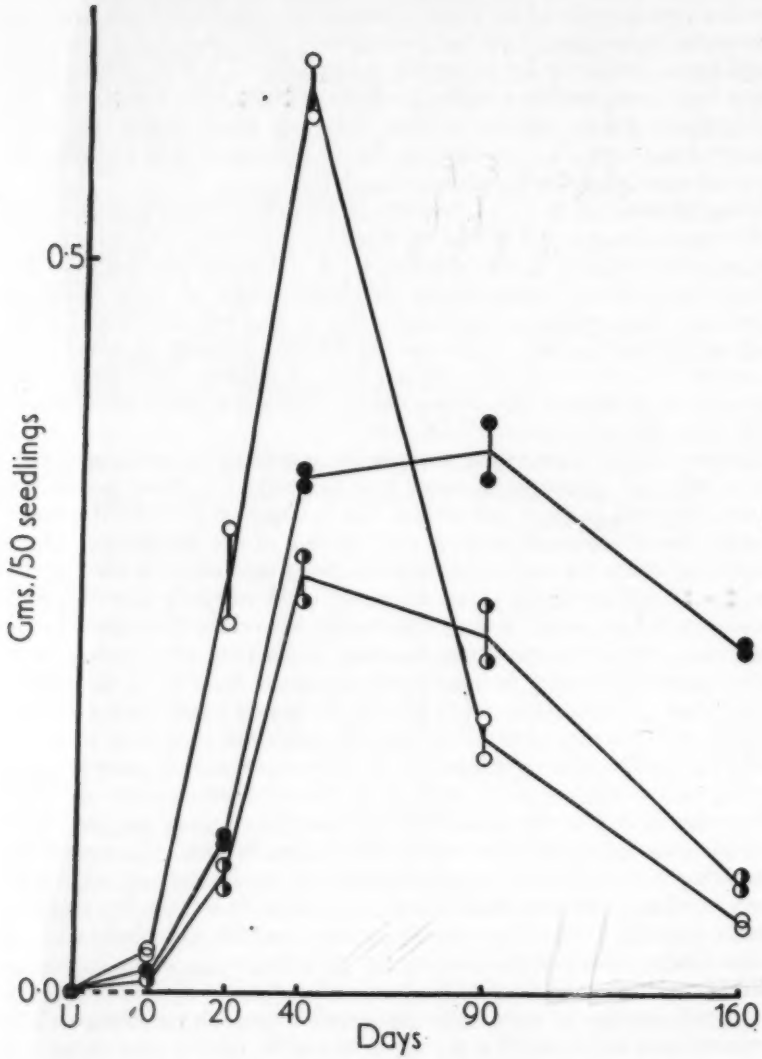


FIG. 3. Fatty acid changes in the haustorium.
○—○, shorter-chain saturated acids (C_8 to C_{14})
◑—◑, longer-chain saturated acids (C_{16} to C_{24})
●—●, unsaturated acids (oleic and linoleic).

oil palm too, since re-esterification accompanies breakdown. However, it seems more probable that there is a genuine difference in the rate of breakdown of longer- and shorter-chain saturated acids (or of higher and lower molecular-weight glycerides) since if each glyceride molecule attacked was completely utilized, although lauric acid might disappear at a faster relative rate (owing to the occurrence of dilaurins), the remaining shorter-chain acids (C_8, C_{10}, and C_{14}) should, on this basis, behave similarly to the longer-chain acids.

The oil-palm haustorium, considered as a fat-catabolizing organ, presents an interesting contrast with *Citrullus* (Crombie and Comber, 1956), since in the cotyledons of the latter the fatty acids which are broken down are those characteristic for the tissue, whereas in *Elaeis* unusual and uncharacteristic fatty acids are continuously being absorbed and metabolized. In both plants the changes in fat composition during breakdown are small in comparison with the large quantities metabolized. In *Citrullus*, palmitic, stearic, oleic, and linoleic acids disappear at approximately constant proportionate rates and the same acids in *Elaeis* form a group which is metabolized at a slower rate than shorter-chain saturated acids (C_8 to C_{14}). No comparison on the rates of breakdown of the last group of acids is possible between *Elaeis* and *Citrullus*, since such acids do not occur in the latter plant.

ACKNOWLEDGEMENTS

We thank Professor W. T. Williams for his interest in this work and the Director and staff of the West African Institute for Oil Palm Research for many generous gifts of seeds.

We are grateful to Miss E. E. Hardman for the analysis of oil-palm embryo fat and for the results of unpublished experiments on oil-palm respiration and lipase action. One of us (S. G. B.) acknowledges with thanks the receipt of an extended open scholarship from the University of Southampton.

LITERATURE CITED

Anon. (1956). Notes on the Botany of the Oil Palm. 2. The seedling. *J. West African Inst. for Oil Palm Research*, **2**, 92–95.

Boatman, S. G. (1956). *Fatty acid metabolism in the developing seedling of* Elaeis guineensis *Jacq.* Ph.D. Thesis, University of Southampton.

Borgström, B. (1951). On the mechanism of intestinal fat absorption. Pts. 3 and 4. *Acta Physiol. Scand.* **25**, 140–9. 291–314.

Carsten, H. A., Hilditch, T. P., and Meara, M. L. (1945). The component acids of the testa and kernel fats of the oil palm. *J. Soc. Chem. Ind.* **64**, 207–9.

Crombie, W. M. (1956). Fat metabolism in the West African Oil Palm (*Elaeis guineensis*). 1. Fatty acid formation in the maturing kernel. *J. Exp. Bot.* **7**, 181–93.

—— and Boatman, S. G. (1955). Reversed-phase chromatography as a method for the analysis of the fatty acids in palm oil and palm kernel oil. *J. West African Inst. for Oil Palm Research*, **1**, 64–69.

—— and Comber, R. (1956). Fat metabolism in germinating *Citrullus vulgaris*. *J. Exp. Bot.* **7**, 166–80.

Desveaux, R., and Kogane-Charles, M. (1952). Étude sur la germination de quelques graines oléagineuses. *Ann. Agron.* **3**, 385–416.

Green, D. E. (1954). Fatty acid oxidation in soluble systems of animal tissues. *Biolog. Revs.* **29**, 330–66.

HENRY, P. (1951). La germination des graines d'Elaeis. *Rev. Int. Bot. Appliq. d'Agric. Trop.* **31**, 566–91.

HOUGET, J. (1942). Sur la formation des glucides au cours de la germination de Ricin. *Compt. rend. (Paris)*, **215**, 387–8.

—— (1943). Sur la mécanisme de la transformation des lipides en glucides au cours de la germination de Ricin. Ibid. **216**, 821–2.

HUMPHREYS, T. E., NEWCOMB, E. H., BOKMAN, A. H., and STUMPF, P. K. (1954). Fat metabolism in higher plants. 2. Oxidation of palmitate by a peanut particulate system. *J. Biol. Chem.* **210**, 941–8.

KIRKWOOD, J. E., and GIES, W. J. (1902). Chemical studies of the coconut with some notes on the changes during germination. *Bull. Torrey Bot. Club.* **29**, 321–59.

LONGENECKER, H. E. (1939). Deposition and utilization of fatty acids of low molecular weight; and a fatty acid analysis of coconut oil. *J. Biol. Chem.* **130**, 167–77.

MURLIN, J. R., MURLIN, W. R., and WATKEYS, J. D. (1933). The conversion of fat to carbohydrate in the germinating castor bean. *J. Gen. physiol.* **17**, 283.

REISER, R. (1954). Some recent studies on fat digestion and absorption. *J. Amer. Oil Chemists' Soc.* **31**, 292–5.

RHINE, J. B. (1926). Translocation of fat as such in germinating fatty seeds. *Bot. Gaz.* **82**, 154–69.

STUMPF, P. K. (1956). Saturated fatty acid peroxidase. *Fed. Proc.* **15**, 366.

WINTON, A. L., and WINTON, K. B. (1932). *The structure and Composition of Foods.* Vol. 1. John Wiley and Sons Inc. New York. Pp. 367–72.

YAMADA, M. (1955). Studies on fat metabolism in germinating castor beans. 2. *Sci. Papers of the College of General Education (Univ. Tokyo)*, **5**, 161–9.

YAMPOLSKY, C. (1922). A contribution to the study of the oil palm. *Bull. Jardin Bot. Buitenzorg, Ser.* 3, **5**, 107–74.

ZIRM, K. L., PONGRATZ, A., and POLESOFSKY, W. (1955). Beitrag zur Konstitution pflanzlicher Lipoide, im besonderen der Lipoidkomponente des Chloroplastins. *Biochem. Z.*, **326**, 405–12.

Submicroscopic Structure of the Cereal Starch Grain

CLARENCE STERLING[1] AND B. J. SPIT

Department of Food Technology, University of California, Davis, California, U.S.A., and Division of Electron Microscopy, Technical Physics Department TNO and TH, Delft, Holland

WITH FOUR PLATES

Received 10 April 1957

SUMMARY

The submicroscopic structure of the starch grains of *Zea mays* and *Triticum sativum* was studied electron-optically from replicas made of internal, fracture surfaces. In corn starch, long cylindrical microfibrils were found, arranged in a radial direction and imbedded in an amorphous matrix. Their diameter was uniformly about 200 Å. Microfibrils were also indicated in wheat starch because of the prominence of their ends in surface view. Many microfibrils in corn starch appeared to be helically coiled. The more ordered starch substance was presumed to be localized principally in the microfibrils.

INTRODUCTION

RECENT efforts to detect a submicroscopic, fine structure in the native grain of starch via electron microscopy have been notably unsuccessful. In 1952 Frey-Wyssling claimed to see visible strands in the residual sacs of gelatinized potato starch. These were said to have a diameter of about 100 Å. However, the structure was neither so uniform nor so distinct that it could be unequivocally accepted as representing any particular feature of the native starch grain. Likewise, Mühlethaler (1955), using ultra-thin sections of starch grains, was unable to detect a definite organization beyond concentric lamellation. Accordingly, Frey-Wyssling (1952, 1953) characterized the fine structure of the starch grain as amicroscopic.

Whistler *et al.* (1955) have also studied ultra-thin sections of starch grains and similarly have had little success in demonstrating a definite structure. The fine granulation seen by Whistler and Turner (1955) and interpreted as lamellar fine structure is not convincingly different from the 'amicroscopic' structure of Frey-Wyssling. Likewise, their 'thin bead-like strands' are difficultly distinguishable as micelles. After heating and lyophilizing suspensions of starch, Guilbot and Levavasseur (1954) have found bizarre structures with little evidence of a fibrillate or other fine texture.

On the assumption that the starch grain has not been properly treated to

[1] The present study was conducted in Delft, where the senior author enjoyed the use of laboratory facilities provided by the hospitality of Professor P. A. Roelofsen of the Laboratory for General and Technical Biology, Technische Hogeschool. He is most grateful for this friendly reception and for the financial aid of the U.S. Educational Foundation in the Netherlands.

reveal its fine structure, the present writers undertook another approach to sample manipulation and preparation for electron microscopy. The results of this investigation constitute the subject of the present paper.

EXPERIMENTAL

An analysis of the procedures of the earlier studies indicated that, if there were a fine structure of electron-microscopic dimensions in the starch grain, this fine structure either had been destroyed by heating or was so constituted that differences in electron density were not to be found in adjacent structural elements. It was decided, therefore, to use a replica method to obtain the configuration of internal surfaces of the starch grain. Also, it was proposed to rupture that grain so that internal fracture surfaces should be accessible to the replica material.

Because of the recognized softness and plasticity of the substance of starch grains (cf. Lepeschkin, 1921; Siostrom, 1936; Badenhuizen, 1937; Alsberg, 1938; &c.), because of the solubility of smaller starch molecules in water, and because of the possibility of residual amylase activity in the sample, the use of liquid water was avoided during sample rupturing. One method of preparation, used for corn starch alone, was to freeze the grains in water at −20° C. The block of ice, with the embedded grains, was then hammered thoroughly, while still frozen, in a cup-shaped metal dish in a room for freezing storage (cf. Scott *et al.*, 1956). The fragments of ice were then placed in a surplus of 96 per cent. ethanol held at −5° C. and so stored until used.

Another method of preparation involved the preliminary dehydration of the starch grains in successive solutions of absolute ethanol and acetone. The grains were then placed in xylene (through several changes) and crushed in that medium. The manner of fracturing was varied: pressing with a flattened rod of stainless steel, rubbing the grains with a glass cover slip with a continuous rotary motion, or rolling an agate pestle back and forth over the grains.

Whatever the method of fragmentation, the grains were next placed on a glass slide and the alcohol or xylene permitted to evaporate. Replicas were made according to the platinum shadow plus carbon layer method described by Dalitz (1953) and by Roelofsen *et al.* (1953). Following the removal of the carbon layer with its attached starch grain fragments from the slide, the starch substance was dissolved with 30–40 per cent. chromic acid. After being washed in distilled water, the carbon films with the shadow material were mounted on specimen grids. (As indicated by Roelofsen *et al.*, 1953, a positive replica is so produced.) The preparations were viewed with Philips EM 75 or EM 100 electron microscopes.

If frozen, hammered starch material is used, it is advisable to shadow the preparation immediately after alcohol evaporation or to keep it quite dry—otherwise the fragments gradually become liquefied. (This phenomenon also occurs if the starch grain has been ruptured in water.) The liquefaction is probably explicable in terms of the adsorption of atmospheric water by the

hygroscopic fragments and the subsequent activity of amylases in the newly formed layer of moisture. This does not occur when the fragments have been produced in xylene. Perhaps there are traces of lipophilic impurities in the xylene which form a thin hydrophobic coating over the granule surfaces.

The samples used in the present study came from specimens of the starch of *Zea mays* and *Triticum sativum* in the museum of the Laboratory for General and Technical Biology of the Technische Hogeschool of Delft. Careful microscopic examination revealed no noticeable evidence of cell-wall fragments or other contaminants in the starch. In addition, the frequency of the EM figures, as shown here, was such that it is improbable that they represent contaminants.

RESULTS

The characteristic appearance of the starch material which is visible after hammering is represented in Figs. 1 and 2. Occurring in elongated aggregations of various sizes, and sometimes singly, are small rodlets, which lie parallel to each other within an aggregation. The rodlets have a uniform diameter of approximately 200 Å but vary in length from 200 to 3,000 Å. (Their general appearance closely resembles that of particles produced in the hydrolysis of cellulose, Rånby, 1952.) The aggregations may occur isolated or they may be associated with amorphous masses, as shown in Figs. 1 and 2. The long axis of the rodlets is parallel to the long axis of the aggregation.

It does not seem reasonable to relate these rodlets to amylose crystals which are precipitated from solution by alcohol. In the first place, their diameter is quite uniform. Secondly, it will be recalled that there has been no opportunity for such a solution to be formed: until the addition of the excess 96 per cent. ethanol, the intact starch grain was frozen in a block of ice or existed as frozen fragments in small slivers of ice during and after the hammering. It is possible that during the evaporation of the alcohol there occurred an aggregation of the short rodlets which were already present as fragments in the ruptured starch mass. However, although it might be expected that the aggregation within a grouplet could occur in a uniform direction, a grouplet would not be expected to tend to have a constant girth along its length.

Another problem with the interpretation of these frozen, hammered fragments is that it is difficult to indicate a definite direction for the rodlets in the intact granule or even to know if one exists. Because of the polarization-optical findings of many workers (e.g. Frey-Wyssling, 1940*a*, *b*; Speich, 1942, &c.), it seems reasonable to assume that the major axes of the rodlets originally lay in a radial orientation.

The presence of the larger groups of rodlets with a preferred orientation (Fig. 2) suggests that these may have been split directly from the starch grain. The presence of longer rodlets also suggests that the short rodlets are fragments of longer ones (a viewpoint which gains support from the aspects of starch grains ruptured in xylene and from the uniform diameter and non-uniform length of the rodlets). Some of these units have an aspect very

suggestive of a helically wound, rope-like structure (see particularly the two shaded regions of Fig. 1, one region being indicated by an arrow in that figure). This aspect is also characteristic of the fracture surface of grains which have been crushed in xylene.

The method of crushing the starch grain in xylene yielded few direct views of an internal surface. Despite its being dehydrated, the starch substance was still sufficiently plastic so that the grain was often greatly deformed before being ruptured. Nevertheless, some grains (particularly those of corn starch) did present their fracture surfaces to the carbon film. The structure of such a surface is best seen in Figs. 3 and 4. These indicate that the starch substance is organized into 'microfibrils' of about 200 Å diameter and apparently indefinite length. Hence, the rodlets described above very likely represent short segments of these microfibrils.

As shown by Noda and Wyckoff (1951) for collagen reprecipitated at a low pH, many starch microfibrils appear to be helically wound. Occasionally they may combine to form a larger rope-like strand, composed of 2 (principally) to 5, or occasionally more, microfibrils. The relationship between the straight rod-like units of Figs. 1 and 2 and Fig. 4, top right, and the helical structures cannot be decided here. It appears that the convoluted forms running from the lower left to upper right in Fig. 4 are caused by shear. Although very likely not determinative for such a large body as the microfibril here, it is suggestive that various workers (Bear, 1942; Caesar and Cushing, 1941; Kreger, 1951; and others) view the starch molecule as being coiled in the crystalline micelle. Similarly, Staudinger and Eilers (1936) among others have proposed a helical form for the starch molecule in solution. To what extent the methods of preparation are responsible for differences in the size and form of the microfibrils can only be conjectured.

In the crushed grain, the microfibrils are quite close together—some being in contact while others may be as much as 50 Å apart. It is to be noted that there appears to be an amorphous ground substance in which the microfibrils are imbedded. Pit-like depressions in the centre of Fig. 4 attest to the prior presence of microfibrils on the surface of these depressions—probably pulled away in the fracturing of the starch grain. Also in Fig. 3 such amorphous regions may be seen. It is of interest to recall the blocklet-cementing substance of Hanson and Katz (1934), but more pertinent still is the idea of Lepeschkin (1921), that the soft crystallites of the starch grain are glued together by a mass of amorphous polysaccharide. (See also Katz, 1933, for a similar concept.)

The general direction of the microfibrillar strands seems to be radial (Figs. 3, 4). Thus far it has not been possible to detect differences in microfibrillar arrangement corresponding to concentric laminations in the starch grain.

Although Whistler *et al.* (1955) have stated that the sectioned starch grain has a smooth surface, it is quite possible that their grains were still covered by the very thin plastid. No statement is made in analysis of the surface replicas obtained by them. Probable surface views of the starch grain exterior are here shown in Figs. 5 and 6. Fig. 6 indicates how readily the starch substance flows

upon shearing. Fig. 5 shows, by virtue of the presence of a more or less papillate texture in the surface of the grain, that the surface tends to be organized into units of about 200 Å in diameter, which corresponds to the size of the microfibrils seen internally and to the diameter of the rodlets noted above. Possibly the very warty protuberances of Fig. 6 and of the central grain of Fig. 5 represent the ends of microfibril groups which come into prominence with relatively small stresses—by virtue of flow of the amorphous material between. These figures indicate again that the microfibrils of both wheat and corn starch are radially arranged.

DISCUSSION

The term, microfibril, was first advanced by Frey-Wyssling in 1937 for the as yet invisible units in which cellulose micelles of the cell wall of fibres were considered to be organized. Although the estimated diameter of the microfibril (which was indicated diagrammatically) was somewhat larger than that subsequently found, the validity of the speculation has since been abundantly verified in many electron-optical studies of the cell wall. The basis for the concept was the finding that metallic salts could be reduced within the cell wall, with the subsequent deposition of metallic crystals of a certain size range. Hence micro-capillary spaces were indicated in the body of wall substance. On the basis of other considerations, that body was conceived to be composed of elongated, rod-like units. Frey-Wyssling (1948) has also suggested that radial, submicroscopic capillary spaces exist in the starch grain.

Such a concept for starch was criticized, however, by Badenhuizen (1939), who stated, 'It has not been proved that the starch substance includes a similar system of intermicellar spaces, as found in cellulose. . . . The regular deposit of small metal crystals after reduction of the salts is an artifact, telling us nothing about a possible existence of submicroscopical spaces.' Although Meyer (1895) also believed that the starch grain had a pore system, some of his findings fit in better with the picture of the grain given here. Thus, after a 3-month treatment of potato starch with malt, fine points appeared on the surface of the grains. After a 5-year treatment with dilute sulfuric acid, although much substance was lost, the polarization figure of potato starch was brighter than that of the normal, untreated starch. Both findings point to attack of the enzyme or acid on the amorphous, inter-microfibrillar starch.

One particularly puzzling datum in starch grain behaviour is the great amount of rapid swelling which takes place in cold water at the site of an injury to the grain (Sjostrom, 1936; Alsberg, 1938; Badenhuizen, 1939). If it be assumed that the starch of the amorphous matrix and the starch of the injured microfibrils be less well ordered than the starch of the intact microfibrils, then water could be taken up more readily in this portion. In the normal grain, the microfibrillar skeleton would perhaps permit only a restricted volume change to occur. If this structure were disrupted, resistance due to the structural configuration would be diminished or lost. Thereby would be permitted an unlimited expansion of the amorphous and disrupted microfibrillar starch

—even some dissolution—at the site of the injury and a gradually decreasing swelling toward the more intact regions, as has been observed (Alsberg, 1938).

Very likely both the microfibrils and the amorphous matrix are quite soft. When the starch grain is compressed, plastic deformation and gradual loss of birefringence are the usual consequences. Frey-Wyssling (1940*a*, *b*) has shown that, after such compression, the molecules are thrown into disorder. Consequently, he has suggested that crystalline micelles are lacking in starch grains and that the birefringence of a starch grain is due only to form effects and tensions in that structure. However, the work of many authors on X-ray diffraction patterns of native starch definitely indicates that a crystal lattice, hence micellar structure, exists. The disappearance of the X-ray pattern of starch on grinding has been ascribed by Sponsler (1922) to the plasticity of the starch crystallites.

The findings presented in this report also explain the lack of positive results in other electron-microscopic studies of starch grain organization. If the only difference between the starch substance of the microfibril and the starch substance of the amorphous matrix is in degree of organization, it is to be expected that for such a material as starch the electron density of both components will be quite similar. Hence, ultra-thin sections will show little detail of the microfibrillar structure as against that of the amorphous substance. Moreover, if the microfibrils tend to be helically wound, thin sections would show a string-of-beads organization in the radial direction, as actually so described by Whistler and Turner (1955). On the other hand, heating of the starch grain in water disperses not only the amorphous starch but also the microfibrillar starch. (The crystalline form of native starch appears to have a loose, little-resistant type of hydrogen bonding since its X-ray diagram disappears on heating, Katz, 1928). Therefore the preparations of Frey-Wyssling (1952) and Guilbot and Levavasseur (1954) cannot be expected to show the microfibrillar structure.

These experiments have indicated the existence of long, rod-like microfibrils, radially arranged and imbedded in an amorphous matrix in the normal starch grains of corn and wheat. Whether such a construction is to be found in the starch of other species of plants (and in the waxy starches) must await further studies of those materials. It is herewith suggested that the method of replicas of internal fracture surfaces should be the method of choice in the study of the organization of the starch grain and perhaps in the study of other biological materials.

LITERATURE CITED

ALSBERG, C. L. (1938). Structure of the starch granule. *Plant Physiol.* **13**, 295–330.

BADENHUIZEN, N. P. Jr. (1937). Die Struktur des Stärkekorns. *Protoplasma*, **28**, 293–326.

—— (1939). Growth and corrosion of the starch grain in connexion with our present knowledge of the microscopical and chemical organization. Ibid. **33**, 440–68.

BEAR, R. S. (1942). The significance of the 'V' X-ray diffraction patterns of starches. *J. Amer. Chem. Soc.* **64**, 1388–92.

CAESAR, G. V., and CUSHING, M. L. (1941). The starch molecule. *J. Phys. Chem.* **45**, 776–90.

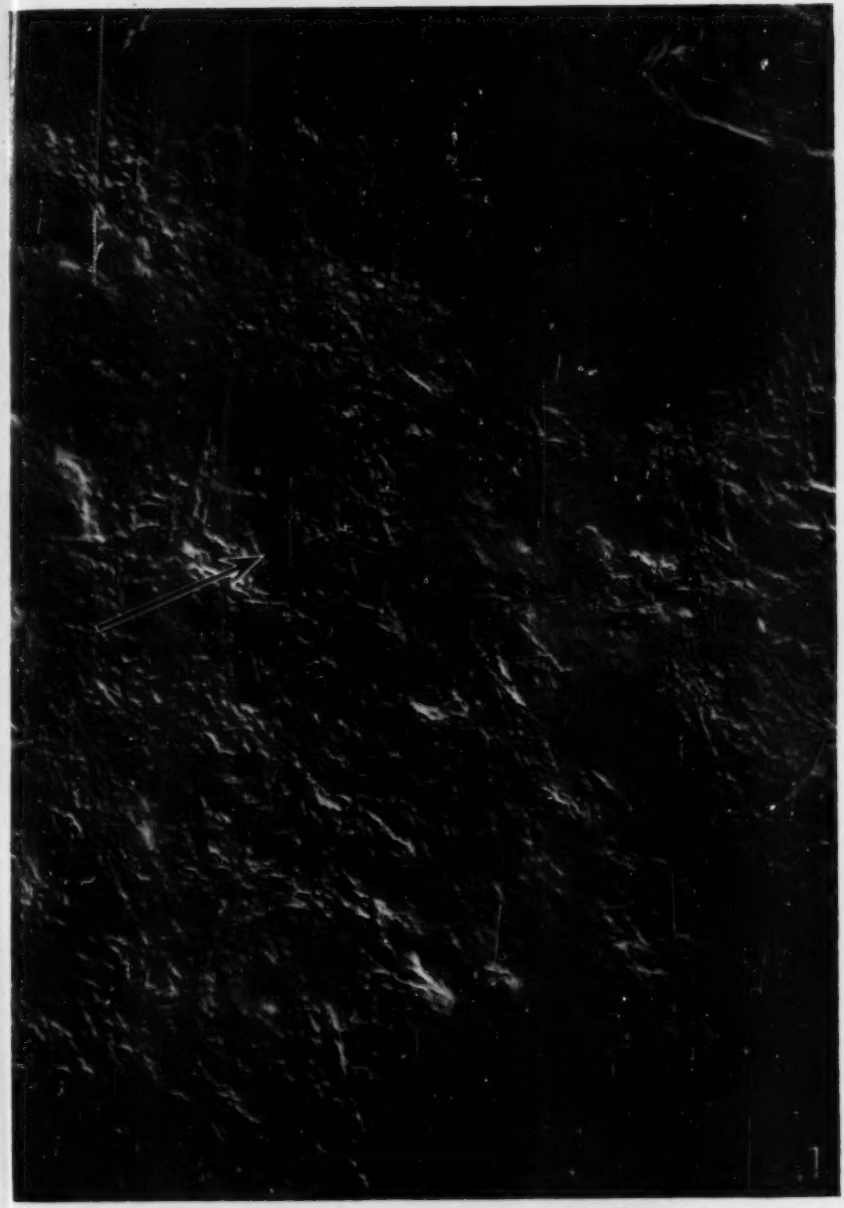


FIG. 1. Aggregations of rodlet fragments of corn starch. Arrow at left centre points to coiled rodlets in shadow region. ×20,000.

C. STERLING *and* B. J. SPIT—PLATE I

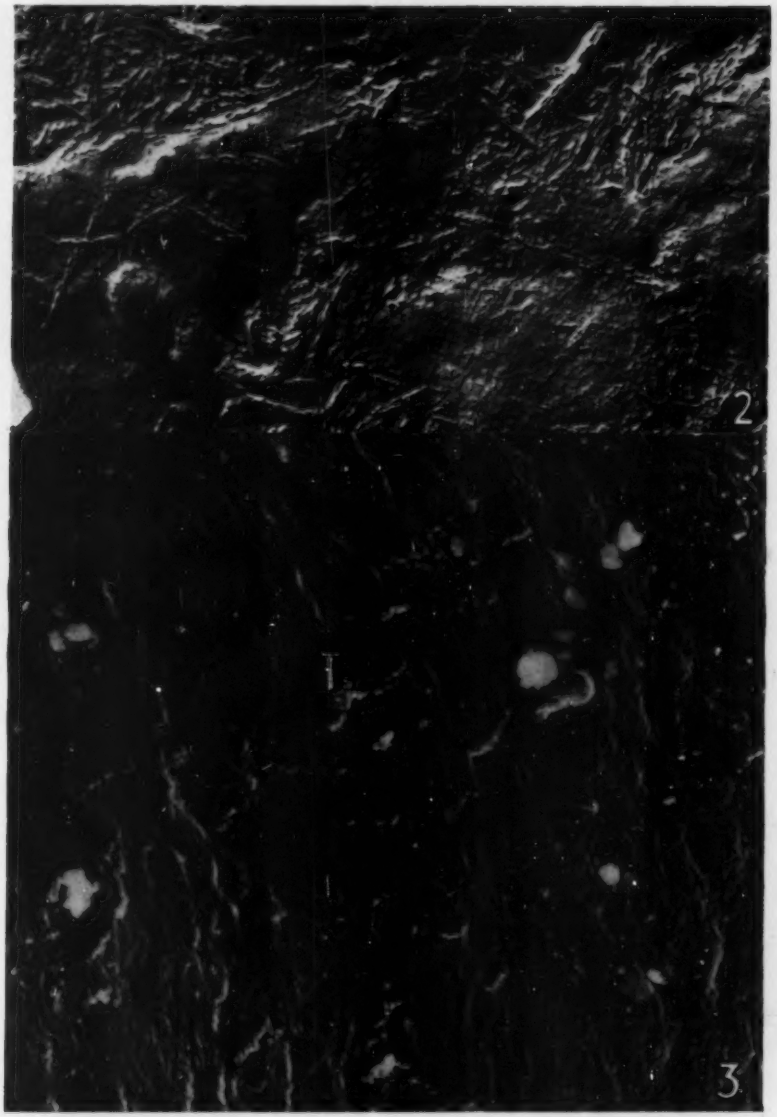


FIG. 2. Rodlet fragments of corn starch in more discrete elongated groups than those of Fig. 1. ×20,000.

FIG. 3. Internal fracture surface of grain of corn starch. Edge of starch grain at top of figure. Fibrillar units appear to be imbedded in amorphous ground substance. ×25,000.

C. STERLING *and* B. J. SPIT—PLATE II

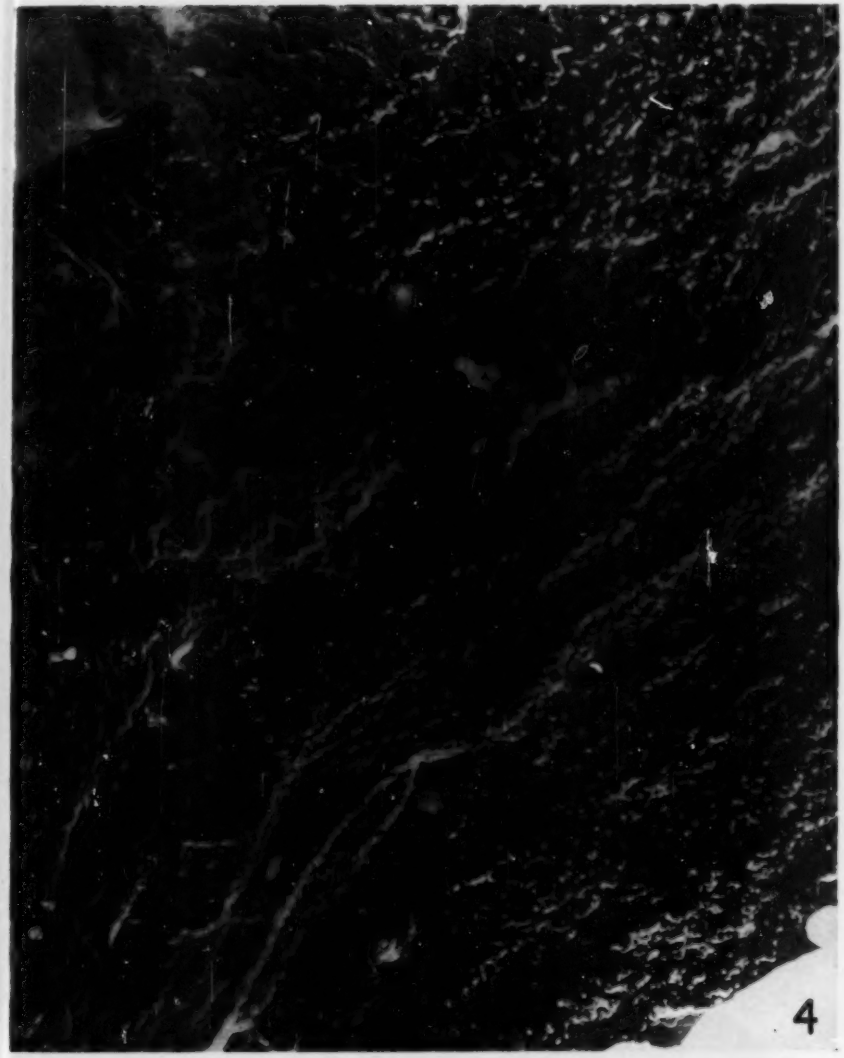


FIG. 4. Internal fracture surface of grain of corn starch. Edge of starch grain at top of page. Note pit-like depressions at lower left and more or less straight, radially directed micro-fibrils at upper right. Compound, helically wound units also at lower left. ×25,000.

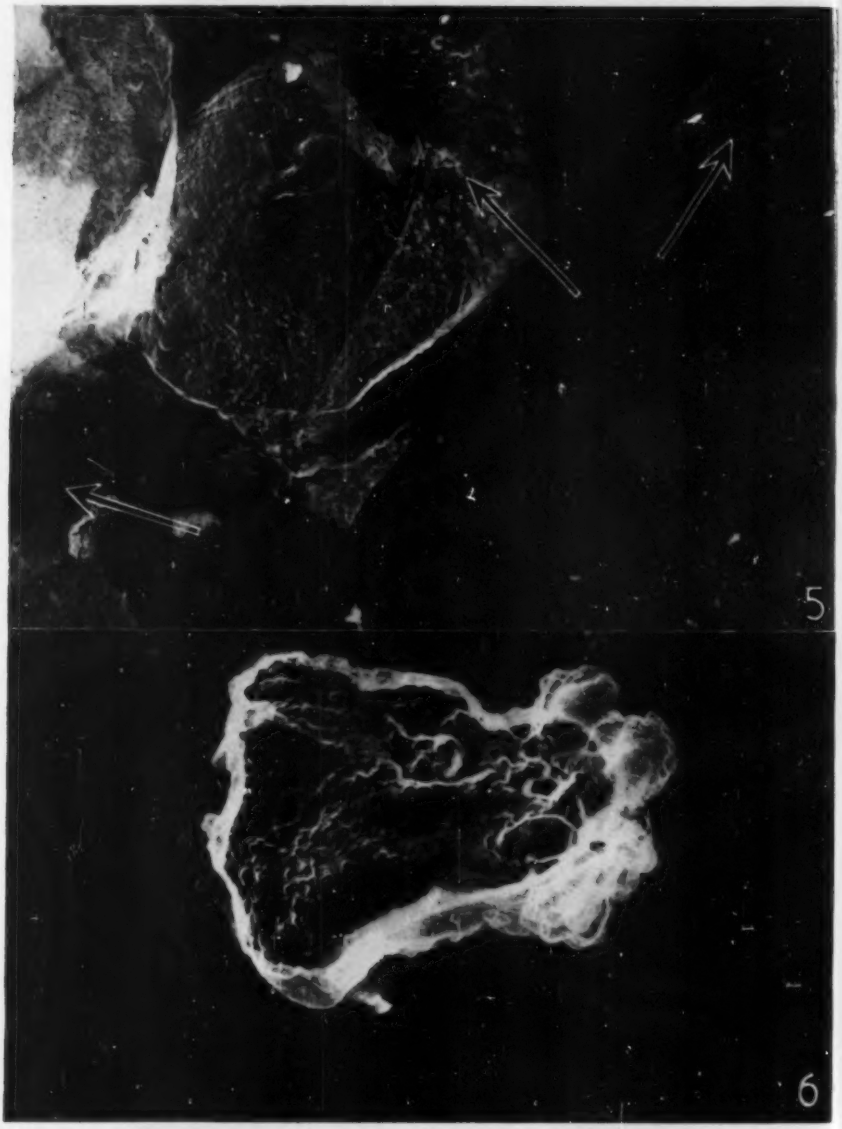


FIG. 5. Surface views of portions of more or less compressed starch grains of wheat. Grains at lower left and upper right are more or less undamaged, and arrows indicate their papillate surfaces. Central grain is somewhat crushed and shows more warty protuberances at surface (at arrow). ×20,000.

FIG. 6. Portion of surface of somewhat compressed starch grain of corn. Warty protuberances are quite prominent. ×20,000.

C. STERLING *and* B. J. SPIT—PLATE IV

DALITZ, V. C. (1953). Abdruckverfahren zur Beobachtung von Holz im Übermikroskop. *Z. wiss. Mikr.* **61**, 292–3.

FREY-WYSSLING, A. (1937). Röntgenometrische Vermessung der submikroskopischen Räume in Gerüstsubstanzen. *Protoplasma*, **27**, 372–411.

—— (1940*a*). Zur Optik der Stärkekörner. *Naturwiss.* **28**, 78–79.

—— (1940*b*). Die Optik der Stärkekörner. *Ber. Schweiz. Bot. Ges*, **50**, 321–36.

—— (1948). Über den submikroskopischen Aufbau der Stärkekörner. *Schweiz. Brauerei-Rundsch.* **59**, 1–5.

—— (1952). Feinbau der Membran gequollener Stärkekörner. *Experientia*, **8**, 101.

—— (1953). *Submicroscopic Morphology of Protoplasm.* Amsterdam.

GUILBOT, A., and LEVAVASSEUR, G. (1954). Structure submicroscopique de l'amidon: I. Mise en évidence, au microscope électronique, de différents éléments structuraux, après dispersion aqueuse du grain à diverses températures. *C. R. Acad. Sci. Paris*, **239**, 728–30.

HANSON, E. A., and KATZ, J. R. (1934). Abhandlungen zur physikalischen Chemie der Stärke und der Brotbereitung. XVIII. Weitere Versuche, die gewachsene Struktur des Stärkekorns mikroskopisch sichtbar zu machen. *Z. physik. Chem.* **169**, 135–42.

KATZ, J. R. (1928). The X-ray spectrography of starch. *A Comprehensive Survey of Starch Chemistry.* (R. P. Walton), **1**, 68–76, New York.

—— (1933). The laws of swelling. *Trans. Faraday Soc.* **29**, 279–300.

KREGER, D. R. (1951). The configuration and packing of the chain molecules of native starch as derived from X-ray diffraction of part of a single starch grain. *Bioch. Biophys. Acta*, **6**, 406–25.

LEPESCHKIN, W. (1921). Étude sur les réactions chimiques pendant le gonflement de l'amidon dans l'eau chaude. *Bull. Soc. Bot. Genève* II. **13**, 40–64.

MEYER, A. (1895). *Untersuchungen über die Stärkekörner.* Jena.

MÜHLETHALER, K. (1955). Untersuchungen über den Bau der Stärkekörner. *Z. wiss. Mikr.* **62**, 394–400.

NODA, H., and WYCKOFF, R. W. G. (1951). The electron microscopy of reprecipitated collagen. *Bioch. Biophys. Acta*, **7**, 494–506.

RÅNBY, B. G. (1952). *Fine structure and reactions of native cellulose.* Dissert. Univ. of Uppsala.

ROELOFSEN, P. A., DALITZ, V. C., and WIJNMAN, C. F. (1953). Constitution, submicroscopic structure and degree of crystallinity of the cell wall of *Halicystis Osterhoutii*. *Bioch. Biophys. Acta*, **11**, 344–52.

SCOTT, F. M., HAMNER, K. C., BAKER, E., and BOWLER, E. (1956). Electron microscope studies of cell wall growth in the onion root. *Amer. J. Bot.* **43**, 313–24.

SJOSTROM, O. A. (1936). Microscopy of starches and their modifications. *Ind. Eng. Chem.* **28**, 63–74.

SPEICH, H. (1942). Über die Optik der Kartoffelstärkekörner. *Ber. Schweiz. Bot. Ges.* **52**, 175–214.

SPONSLER, O. L. (1922). The structure of the starch grain. *Amer. J. Bot.* **9**, 471–92.

STAUDINGER, H., and EILERS, H. (1936). Über hochpolymere Verbindungen. 136. Mitteilung: Über den Bau der Stärke. *Ber. d. d. chem. Ges.* **69**, 819–48.

WHISTLER, R. L., BYRD, J. D., and THORNBURG, W. L. (1955). Surface structure of starch granules. *Bioch. Biophys. Acta*, **18**, 146–7.

—— and TURNER, E. S. (1955). Fine structure of starch granule sections. *J. Polymer Sci.* **18**, 153–6.

The Stimulatory Effect of Indole-3-Acetic Acid on the Uptake of Amino-Acids by Tissue of *Helianthus annuus*

LEONORA REINHOLD AND R. G. POWELL

Agricultural Research Council Unit of Experimental Agronomy, Department of Agriculture, University of Oxford

Received 8 July 1957

SUMMARY

Indole-3-acetic acid was observed to bring about a prompt and marked increase in the amount of ^{14}C accumulated by segments of sunflower hypocotyl from solutions of labelled glutamic acid, glycine, and lysine. The curve relating magnitude of effect to indole-3-acetic acid concentration followed the comparable curves for water uptake and extension growth.

The accumulation of ^{14}C was related to the external concentration of glutamic acid by a curve which departed only slightly from linearity. The percentage increase in ^{14}C accumulation brought about by auxin did not decline to any appreciable extent with increasing external concentration of glutamic acid.

Under nitrogen the amount of ^{14}C taken up from solutions of labelled glutamic acid in 1·75 hour was cut down by approximately one-third, and the auxin effect was abolished. The Q_{10} for ^{14}C accumulation between 16° C. and 26° C. was 1·2 in the absence of indole-3-acetic acid, and was 1·3 in its presence.

When net water uptake was eliminated by the addition of mannitol to the external solution, ^{14}C accumulation in auxin-free media was not depressed. The percentage increase in ^{14}C accumulation brought about by auxin, however, was markedly reduced.

The fate of the ^{14}C accumulated was investigated by means of chromatography on resin columns and on filter paper. About 30–40 per cent. of the ^{14}C was in the form of glutamic acid after approximately 2 hours' treatment. No marked difference in the level of glutamic acid was observed between auxin-treated and control segments. The effect of auxin was more evident on the amounts of other radioactive derivatives, as yet unidentified.

It was observed that, not only was the amount of CO_2 evolved in respiration higher in the presence of indole-3-acetic acid, but that this CO_2 was richer in ^{14}C, i.e. in auxin-treated tissue glutamic acid formed a larger proportion of the substrate respired.

The possible implications of these observations are discussed. It is pointed out that indole-3-acetic acid may have achieved its effect by stimulating a transfer process, by lessening a diffusion resistance, or by promoting a process or processes which, by removing free amino-acids within the cell, maintain an inward diffusion gradient.

INTRODUCTION

ALTHOUGH plant growth-regulating substances produce many profound and far-reaching effects on plant cells, reports of effects on animal cells are rare. The finding by Christensen, Riggs, and Coyne (1954) that indole-3-acetic

acid (in relatively high concentration) promotes the accumulation of amino-acids by mouse tumour cells was therefore of particular interest. Little direct evidence is available that indole-3-acetic acid stimulates the uptake of solutes of any description by plant cells. There is no general agreement as to its effect on salt uptake. An enhanced rate of uptake of ions in the presence of growth regulators has, however, been observed by Commoner and Mazia (1942), Higinbotham *et al.* (1953), and Hanson and Bonner (1954), and Henzell produced evidence for an increased absorption of potassium by *Salvinia natans* in the initial period following treatment with 2:4-dichlorophenoxy-acetic acid (see Blackman (1956)). Said and Naguib (1952) reported a stimulatory action of indole-3-acetic acid on the uptake of sucrose by carrot disks. This response, though, was slow to manifest itself, and became marked only after some days. The work to be presented here was undertaken to investigate whether an effect on amino-acid uptake similar to that described by the animal workers could be demonstrated in plant cells. Some of the findings have already been briefly reported elsewhere (Reinhold and Powell, 1956).

MATERIALS AND METHODS

The sunflowers (*Helianthus annuus*) used in this investigation were of the variety Pole Star, and were grown in sterile sand at 24° C., in darkness except for occasional red light. When the seedlings were 6 days old segments 1 cm. long were cut from the hypocotyls at a distance of a centimetre below the crook. The segments were washed in distilled water and subsequently suspended in briskly aerated solutions of radioactive amino-acids, uniformly labelled with ^{14}C. The solutions were buffered to pH 6·0 with a KH_2PO_4—KOH mixture (final concentration 0·02 M) and were maintained at 26° C. After periods varing from 0·5 to 3·5 hours the segments were removed, well rinsed with distilled water, and freeze-dried. Their radioactivity was then determined by a technique based on that suggested by Van Slyke *et al.* (1951). The $BaCO_3$ obtained was counted as an 'infinitely thick' layer.

Measurements of water uptake were included in many of the experiments. For this purpose the segments were carefully blotted with filter paper to remove surface moisture, and weighed on a torsion balance sensitive to 1 mg. Where respiration measurements were included, standard manometric technique was employed unless otherwise indicated.

In certain experiments extracts of the segments were made at the conclusion of the experiment and their radioactive content examined by chromatographic techniques, using both filter paper and 'Dowex-50' columns as described by Moore and Stein (1951). The extracts were made either by grinding the segments in 0·1 N HCl in a glass mortar, or by dropping them into hot alcohol so as to give a final concentration of 85 per cent. In some experiments the alcoholic extracts were concentrated and applied directly to the paper; in others they were evaporated to dryness under reduced pressure, the residue being taken up in dilute alkali, brought to pH 5 with HCl, and applied to the paper or column. The descending paper chromatographic

technique was followed, using Whatman No. 1 paper. The usual running solvent was phenol-water in the proportion 80:20; the temperature was 25° C.

The 'Dowex-50' column was developed with citrate buffer, pH 3·42, at room temperature. At this temperature glutamine can be included in the assay. About 65 per cent. is recovered, together with asparagine in a single peak (cf. Barry, 1956).

Radioactive compounds were located on the paper chromatograms by means of a recording scanner built by Dr. R. P. Martin (see Loughman and Martin, 1957): in the successive fractions of eluent from the column they were detected by a direct-plating technique devised by Dr. C. C. McCready of this Unit. In this method the uniformity of the plate is ensured by incorporating the sample in an agar gel on the planchet, which is subsequently dried under an infra-red lamp. The standard error of this method as applied to column eluate was $\pm 2{\cdot}5$ per cent. Amino-acids were also determined in the eluate by a modification of Moore and Stein's colorimetric technique (1948).

EXPERIMENTAL RESULTS

A marked increase in the amount of ^{14}C incorporated from solutions of glutamic acid and glycine has been observed to occur when indole-3-acetic acid is present in the medium. This is shown in Table I, where three typical experiments have been summarized. It will be seen that the size of the stimulation ranged from 40–70 per cent. The effect has also been noted in preliminary experiments with lysine. This response of the tissue to auxin is a rapid one, being clearly apparent in experiments lasting less than 30 minutes.

TABLE I

The effect of indole-3-acetic acid on the accumulation of carbon-14 from solutions of uniformly labelled amino-acids by segments of sunflower hypocotyl

Experiment	Experimental conditions	IAA (μM.)	Sp. Activity of segments mμc./mM. C*	Per cent. increase in sp. activity
I. Glutamic acid 10^{-3} M.	16 segments† in 3 ml. for 2 hr.	0	10·3 11·1	
		8·6	17·5 19·3	72
II. Glutamic acid $2{\cdot}1 \times 10^{-3}$ M.	12 segments† in 2 ml. for 2·25 hr.	0	53·4 53·8	
		29	81·7 92·3	62
III. Glycine 2×10^{-3} M.	12 segments† in 3 ml. for 2 hr.	0	47·4 43·2	
		29	59·7 69·5	43

* The carbon content of the segments was not perceptibly affected by the treatments.

† The fresh weight of 10 segments is approximately 280 mg.

Further experiments were carried out to investigate how the auxin concentrations which elicit this response compared with those which influence uptake of water and extension growth. Fig. 1 shows that the curves relating magnitude of effect to auxin concentration are very similar for the three processes, rising to a maximum value between $2{\cdot}86\times10^{-5}$ M. and $1{\cdot}43\times10^{-4}$ M., and subsequently declining.

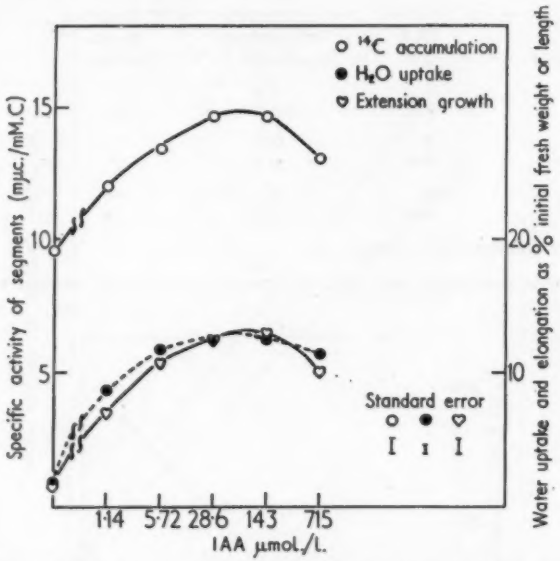


FIG. 1. The effect of concentration of indole-3-acetic acid on the ^{14}C accumulation, water uptake, and extension growth of segments of sunflower hypocotyl suspended in labelled glutamic acid solutions. 12-segment samples (approximately 0·38 g.) were placed in 3 ml. 10^{-3} M. glutamic acid for 2·25 hrs.

Under nitrogen the auxin effect is abolished. This may be seen in Table II, which also shows that, under the conditions of the experiment, anaerobiosis cuts down the accumulation of ^{14}C in the absence of auxin by approximately $\frac{1}{3}$. The temperature coefficient for ^{14}C accumulation between 16° C. and 26° C. appears to be 1·2 in the absence of auxin, and is 1·3 in its presence (Table II).

The relationship between external concentration and ^{14}C accumulation has been investigated for glutamic acid over a concentration range of $2{\cdot}0\times10^{-3}$ M.–$8{\cdot}7\times10^{-2}$ M. Over this range the line relating these two functions shows only a very slight curvature (Figs. 2 and 3).

Several of the observations noted in the last two paragraphs—the shape of the curve relating rate of uptake to external concentration, the relatively low Q_{10}, and the fact that ^{14}C was taken up even under anaerobic conditions—might be taken as indications that the process or processes affecting ^{14}C

TABLE II

The effect of (a) *temperature and* (b) *anaerobic conditions on the accumulation of* ^{14}C *from solutions of labelled glutamic acid by segments of sunflower hypocotyl*

(12-segment samples (approximately 0·4 g.) were placed in 3 ml. $1{\cdot}5 \times 10^{-3}$ M. glutamic acid for 1·75 hrs. Nitrogen was bubbled briskly through the anaerobic media for 25 min. before the introduction of the segments and throughout the experiment.)

Conditions	IAA μM.	Sp. activity of segments (mμc./mM. C)*	% Increase in sp. activity	Q_{10}
Aerated 26° C.	0	7·9	—	1·2
	8·6	12·0	52	1·3
Aerated 16° C.	0	6·6	—	—
	8·6	9·1	38	—
Nitrogen 26° C.	0	5·4	—	—
	8·6	5·3	0	—

Significant difference ($P = 0{\cdot}05$) 1·4

* The carbon content of the segments was not perceptibly affected by the treatments.

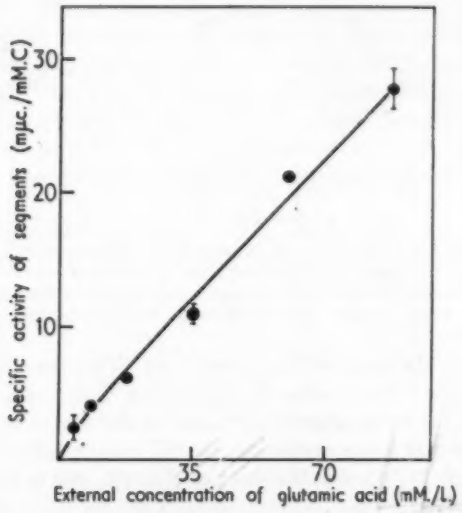


FIG. 2. The effect of external concentration of labelled glutamic acid on ^{14}C accumulation by segments of sunflower hypocotyl. 12-segment samples (approximately 0·34 g.) were suspended in 3 ml. medium for 1·75 hrs. Each point represents the mean of duplicates. The range of the latter is indicated where this extended beyond the symbols drawn.

uptake are predominantly physical in nature. On the other hand, the fact that the amount of ^{14}C accumulated is depressed in the absence of aerobic metabolism, and that the Q_{10} is in excess of 1, suggest that metabolic processes may be

involved as well. The possibility that uptake is partly mediated by a physical process, and partly by a metabolic one, was therefore considered. Since saturation of the enzyme systems concerned would set an upper limit to the rate of the metabolic process, whereas the rate of the physical one would rise indefinitely with external concentration, uptake due to the metabolic process would form a smaller and smaller proportion of total uptake as the external

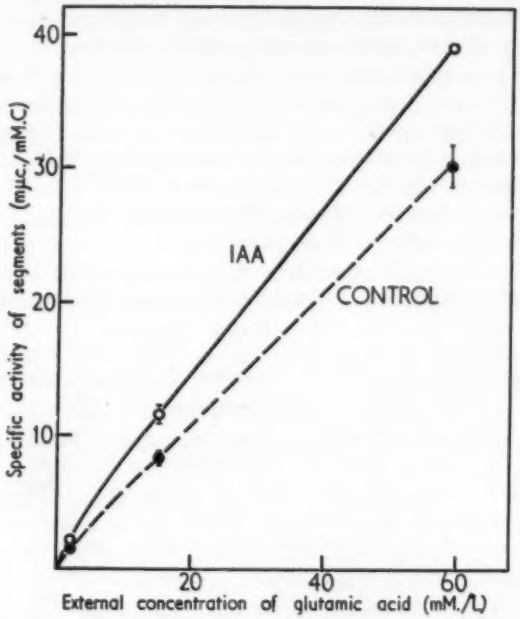


FIG. 3. The effect of indole-3-acetic acid on the accumulation of ^{14}C by segments of sunflower hypocotyl suspended in various concentrations of labelled glutamic acid. 12-segment samples (approximately 0·39 g.) were placed in 3 ml. medium for 1·75 hrs. Concentration of indole-3-acetic acid: $8{\cdot}6 \times 10^{-6}$ M. Each point represents the mean of duplicates. The range of the latter is indicated where this extended beyond the symbols drawn.

concentration was increased. If indole-3-acetic acid promoted one type of process only, therefore, the magnitude of its effect expressed as a percentage of total uptake would be expected to alter with increasing concentration. Fig. 3 summarizes an experiment carried out to investigate this point. It will be noted that the percentage increase in ^{14}C incorporation brought about by the auxin did not decline to any appreciable extent with increasing external concentration of glutamic acid. The actual figures are 38, 39, and 30 per cent. at external glutamic acid concentrations of 2·2, 15·5, and $58{\cdot}8 \times 10^{-3}$ M. respectively. The experiment thus provides no indication that two types of process are involved.

Glutamic acid uptake in the presence of mannitol. In the experiments summarized above the uptake of glutamic acid was accompanied by the uptake of water from the surrounding medium. In the presence of auxin this uptake of water led to a gain in fresh weight of approximately 13 per cent. in the course of a 2-hour experiment. The corresponding figure in its absence was 3–4 per cent. The greater expansion of cell volume in media containing auxin is, however, not sufficient to account for a greater uptake of glutamic acid on the basis of simple diffusion. The ratio of the final volume of the auxin-treated segments to that of the control segments would, on the figures just quoted, be 113:103. The corresponding ratio for ^{14}C accumulation, as may be seen in Table I, is far greater. Possibly, however, the water entering during the course of the experiment has a special status with respect to the diffusion of glutamic acid. In order to find out whether the uptake of glutamic acid and the uptake of water were in any way linked, experiments were carried out in which net water uptake was reduced or eliminated by means of the addition of suitable concentrations of mannitol to the medium.

The results of one such experiment are summarized in Table III. Considering first the figures for the segments not treated with indole-3-acetic

TABLE III

The effect of mannitol in the external medium on the uptake of glutamic acid with and without indole-3-acetic acid (8·6 μM.)

(12-segment samples (approximately 0·4 g.) in 3 ml. uniformly labelled glutamic acid, 10^{-2} M., for 1·5 hrs.)

Sample	Treatment: IAA	Treatment: Mannitol	H_2O Uptake as % initial fresh weight	Sp. activity of segments mμc./mM. C	Per cent. increase in sp. activity
A	−	None	2·7	2·84	66
B	+	None	13·0	4·72	
C	−	0·16 M.	0·5	3·06	35
D	+	0·16 M.	0·9	4·13	
E	−	0·28 M.	−3·5	3·19	28
F	+	0·28 M.	−1·7	4·09	
Significant difference ($P = 0·05$)			1·7	0·45	

acid (samples A, C, and E) it will be seen that, although in the mannitol media water uptake was much reduced—in the more concentrated medium actual loss of water from the tissue occurred—^{14}C accumulation was not depressed, but was on the contrary somewhat enhanced. (Since the mannitol-treated segments were about 6 per cent. higher in total carbon content, the figures in the table, which refer to *specific* radioactivity, if anything underestimate their ^{14}C accumulation.) On the other hand, the values for samples B, D, and F show that ^{14}C accumulation by the auxin-treated segments was lower in the presence of mannitol. The percentage increase in ^{14}C accumulation produced by auxin was thus decidedly reduced in the mannitol media (last column in table).

While a full interpretation of these results must await further study, it appears that, at least in the absence of indole-3-acetic acid, there is no direct connexion between the uptake of water and the uptake of glutamic acid. The reduction in the auxin-induced stimulation of amino-acid uptake is possibly connected with the arrest of cell extension, which may prevent auxin from exerting its full effect.

In further experiments it has been observed that in media where indole-3-acetic acid and mannitol are both present (in concentrations similar to those used in the experiment just described) respiration is about 10 per cent. lower than where indole-3-acetic acid is present alone. This finding recalls the reports by Bonner, Bandurski and Millerd (1953) for Jerusalem artichoke tissue, and by Ordin, Applewhite, and Bonner (1956) for *Avena* coleoptile sections, that with increasing mannitol concentration in the external solution the auxin-induced stimulation of respiration falls, the rate for auxin-treated tissue approaching that for untreated tissue. An enhanced rate of respiration in auxin-free media when mannitol is present, corresponding to the raised rate of ^{14}C incorporation, has not been observed.

The analysis of plant extracts. In order to investigate the fate of the ^{14}C accumulated by the tissue chromatographic techniques were adopted. Extracts of the tissues were made at the end of the experiments and the radioactive compounds were separated both on filter paper and on 'Dowex-50' columns (see Materials and Methods). It was found that only about 30–40 per cent. of the radioactivity of an alcoholic extract made from the plant material at the end of a 2–2·5 hour experiment is in the form of glutamic acid. A record of a typical paper chromatogram is shown in Fig. 4. B is the glutamic acid peak. This has been established by comparing the chromatogram of an aliquot of the plant extract with that of a further aliquot to which ^{14}C-labelled glutamic acid had been added. Glutamine contributes to peak C. Equally prominent with the glutamic peak is one with a lower Rf. (peak A), and it is this peak which is most obviously enlarged by auxin treatment. When peak A is eluted and re-run in butanol-glacial acetic acid-water (40:10:10) it is resolved into 3 peaks of Rf. 0·16, 0·42, and 0·76 respectively. Work on the identification of these peaks is in progress. That they are not artifacts formed from glutamic acid during the extraction in hot alcohol and subsequent procedure (e.g. pyrrolidone-carboxylic acid) has been shown by adding labelled glutamic acid to a hot alcoholic plant extract, which was then subjected to the usual experimental treatment and chromatographed.

An example of a histogram obtained by the use of the 'Dowex-50' column is shown in Fig. 5. This histogram depicts both the radioactivity and the concentration of ninhydrin-positive substances in successive fractions of eluate from the column. The position of the glutamic acid peak has again been established by adding ^{14}C-labelled glutamic acid to an aliquot of the plant extract immediately before it was placed on the column. The most conspicuous effect of auxin is in raising the level of the radioactive peaks II and III. When aliquots of the fractions of eluent corresponding to these peaks are

applied to a paper chromatogram and chromatographed in phenol-water they run to the peak A zone of the paper.

The analyses have not indicated a marked difference in glutamic acid levels between auxin-treated and control plants. The colorimetric determinations revealed that the native level of glutamic acid is very low in this tissue, of the order of 25 μg. per g. fresh weight or less. The glutamine+asparagine content, on the other hand, is much higher—of the order of 3 mg. per g. fresh

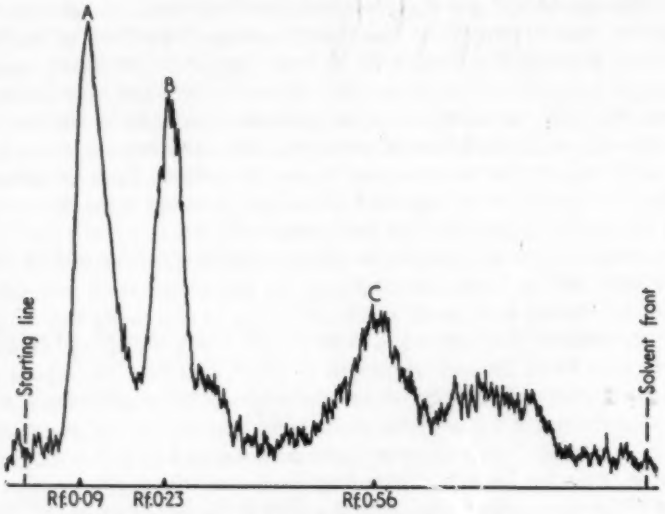


FIG. 4. The distribution of radioactivity on a chromatogram made from an alcoholic extract of treated segments of sunflower hypocotyl. Experimental conditions: 24 segments (0·71 g.) suspended in 4 ml. medium (uniformly labelled glutamic acid $1{\cdot}6\times10^{-3}$ M., indole-3-acetic acid $2{\cdot}9\times10^{-3}$ M.) for 2·2 hrs. Running solvent: Phenol: water (80:20).

weight. A possibility to be considered is that the balance of the reactions involving glutamic acid in these cells is such that the concentration of free glutamic acid is always at a very low level. If this is so, the greater part of the glutamic acid detected in the tissue at the end of an experiment may in fact be recovered from the intercellular spaces and cell walls. Intracellular glutamic acid may form only a small percentage of this total, and the influence of auxin upon it may consequently not be detectable.

The absolute amount of glutamic acid recovered is consistant with the view that it came only from intercellular spaces and walls, and that these areas were in diffusion equilibrium with the external solution. In the experiment from which the histogram in Fig. 5 is taken, the ninhydrin determinations indicated that the final glutamic acid content of the tissue sample was 275 μg. per g. of tissue. The final concentration in the external medium was approximately

5,900 μg./ml. (The concentration of the medium has been found to fall by very few parts per cent. during the course of an experiment.) Thus the amount of glutamic acid in 1 ml. of tissue was only about 5 per cent. of that in 1 ml. of the surrounding medium. It follows that only 5 per cent. of the volume of the tissue could have been in diffusion equilibrium with the external solution.

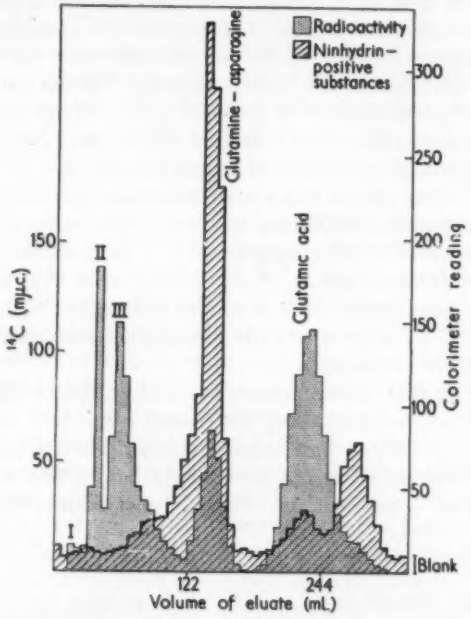


FIG. 5. Histograms representing the concentration of radioactivity and ninhydrin-positive substances separated on a 'Dowex-50' column from an alcoholic extract of treated sunflower hypocotyls. Experimental details: 1·6 g. sample of segments suspended in 9 ml. of medium (uniformly labelled glutamic acid 4×10^{-2} M., indole-3-acetic acid $2{\cdot}86 \times 10^{-5}$ M.) for 2·5 hrs. 85 per cent. of the total extract was applied to the column. Eluent: citrate buffer pH 3·42. Temperature of column: 18° C.

A similar estimate results from a consideration of the figures for radioactivity in another experiment. About 40 per cent. of the total radioactivity of the segments was due to glutamic acid. (This figure was indicated both by measurements of the areas of the peaks on the paper chromatograms and by the analyses of the column eluate.) From the figures for the specific activity of the tissue, its carbon content, and the determination of the enrichment ratio of the glutamic acid in the medium, it was calculated that the final concentration of glutamic acid in the tissue was 340 μg./ml. The concentration in the medium was again 5,900 μg./ml. Thus about 5·8 per cent. of the volume of the tissue could have been in diffusion equilibrium with the medium.

The determination of the specific activity of the ninhydrin-positive substances is subject to a considerable degree of error, on account of the difficulty involved in precisely delimiting the individual radioactive and ninhydrin-positive peaks on the histograms. The values arrived at show no marked effect of indole-3-acetic acid on the specific activity of either glutamic acid or glutamine-asparagine. In the experiment to which Fig. 5 refers the values were: (*a*) Control sample. Glutamic acid: 241 μc. per mM. Glutamine+asparagine: 9·4 μc. per mM. (*b*) Auxin-treated sample. Glutamic acid: 253 μc. per mM. Glutamine+asparagine: 8·8 μc. per mM. (For the purpose of this calculation 139 was taken as the 'molecular weight' of the glutamine-asparagine mixture.) The enrichment of the medium in this experiment was 253 μc. per mM. glutamic acid. There is thus no evidence for any appreciable dilution of the glutamic acid recovered from the tissue.

Respiration. Under the conditions of this investigation the rate of respiration of auxin-treated segments was 20–30 per cent. higher than that of control segments. This effect appeared to be independent of the external concentration of glutamic acid or glycine, or indeed of their presence. In the absence of auxin these amino-acids produced a 10–20 per cent. increase in the respiration rate. The latter rose only very slightly with increasing external concentration of the amino-acids.

A number of further experiments were undertaken to find out whether indole-3-acetic acid, besides raising the overall respiration rate, also altered the specific activity of the carbon dioxide evolved. For this purpose the latter was quantitatively absorbed in CO_2-free NaOH (0·05 N) and was subsequently precipitated as $BaCO_3$ and counted. Table IV summarizes the results of such

TABLE IV

The effect of indole-3-acetic acid (29 μM.) on the quantity and specific activity of CO_2 evolved by segments of sunflower hypocotyl bathed in solutions of uniformly labelled glutamic acid

(11·2 g. tissue samples in 125 ml. 2×10^{-3} M. glutamic acid for 2 hrs.)

IAA	μM. CO_2 evolved	Sp. activity of CO_2 (mμc./mM. C)
−	185	783
	185	750
+	230	936
	247	878

an experiment. The first column shows that the auxin-treated segments evolved 29 per cent. more CO_2 than did the control segments. Furthermore, as may be seen in the second column, the CO_2 produced in the presence of auxin was 18 per cent. richer in ^{14}C. It follows that auxin had brought about a 52 per cent. increase in the amount of glutamic acid respired, while only raising the total rate of respiration by 29 per cent. Thus in the presence of

indole-3-acetic acid, glutamic acid was forming a larger proportion of the substrate respired.

DISCUSSION

It is evident from the experiments reported in this paper that indole-3-acetic acid influenced the amount of amino-acid taken up by the cell from the surrounding medium. There are several possible ways in which it may have achieved this effect. Three suggestions for its mode of action are set out below. The first two propose that it directly affected the process by which amino-acids enter the cell; the third, that it acted indirectly.

(I) Indole-3-acetic acid may have stimulated a transfer process, as is thought to be the case in the animal cells referred to earlier. No such transfer mechanism for amino-acids is definitely known to exist in the plant tissue investigated here. Arisz (1953) reported active transport of amino-acids in certain highly specialized organs, the tentacles of *Drosera capensis*. Evidence for the active accumulation of histidine by root-storage tissue (carrot slices) has recently been produced by Birt and Hird (1956). Webster (1954) found that respiratory inhibitors depress ^{14}C accumulation by bean hypocotyls bathed in solutions of radioactive amino-acids, but this inhibitory action need not have been on an uptake mechanism: the amino-acids may have been able to diffuse readily into the cytoplasm, and the action of the inhibitors may have been on processes which, by consuming free amino-acids within the cell, maintain the inward diffusion gradient (see Proposal III).

(II) If amino-acids penetrate the cell by diffusion against a considerable resistance, indole-3-acetic acid may have brought about a lessening of this resistance. This suggestion requires that the relative rates of diffusion into the cell, and consumption within the cell, are such that diffusion equilibrium with the external solution is not normally established within the experimental period (since, were equilibrium established both in auxin-treated and in control tissue, analyses made at the end of the experiment would not reveal the more rapid attainment of equilibrium in the auxin-treated segments.) Equilibrium may, however, be set up under certain conditions, such as low oxygen tension or the presence of inhibitors, which slow down the rate of the consuming processes. Since net uptake would stop once equilibrium had been established, these conditions would reduce total uptake as measured over the experimental period.

(III) Indole-3-acetic acid may have stimulated one or more of the processes, such as peptide formation or conversion into respiratory intermediates, which remove free amino-acids within the cell and are thus responsible for maintaining an inward diffusion gradient for the acid. Such an influence on the metabolism of amino-acids would have a marked effect on uptake only if the external solution and some region, at least, of the cell interior were at or near equilibrium. If a considerable diffusion gradient inwards existed in any case the effect would be slight.

On the basis of proposal (I) the relation between rate of uptake and external

concentration should be non-linear, the curve flattening out as a limiting value was approached. This is due to the fact that the uptake mechanism is presumably capable of saturation.

In case (III) diffusion into the cell would be directly regulated by the enzyme-catalysed processes which consume free amino-acids within the cell. The ^{14}C detected in the tissue at the end of an experiment would consist of the reaction products of these processes, together with the free glutamic acid recovered from that region of the tissue in diffusion equilibrium with the external solution. One might reasonably expect the curve relating the rate of formation of the reaction products to the external concentration to be a rectangular hyperbola and that for the diffusion component a straight line. The overall curve for uptake would be a combination of these two elements.

Case (II) is similar to case (III), excepting that, owing to the resistance to diffusion, the concentration at the surfaces where consumption occurred would be appreciably different from the external concentration. This would result in the modification of the curve relating the rate of the consumption processes to external concentration into an *oblique* hyperbola (cf. Maskell (1928), Fig. 26). The overall curve for uptake would thus be a combination of a straight line and an oblique hyperbola.

Birt and Hird (1956) have suggested that in carrot disks gross uptake of histidine is mediated by two distinct processes, one physical and one active. (It must be pointed out, however, that a graph constructed from the data they provide in their Table 4, so as to show the relationship between rate of uptake and external concentration, gives no evidence of a physical component, since whether the duration of uptake was 0·5, 1, or 6 hours the curves flatten out completely above a concentration of 25·0 mM. One would have expected that the curve for the 0·5 hour readings particularly would continue to rise with increasing external concentration since, according to the authors' interpretation, uptake during the first 30 minutes is almost entirely due to physical equilibration.) If a similar situation exists in sunflower tissue—and, as has been pointed out on p. 86, the Q_{10} and the results obtained under nitrogen suggest that this may be the case—then the curve expected under proposal (I) would also be modified by a linear component.

The relation obtained experimentally in the present investigation departs only slightly from linearity. The curvature is possibly greater at lower than at higher glutamic acid concentrations, suggesting there may be a combination of linear and curved components; but if this is the case the contribution of the linear component to the overall curve is relatively very large, and if the effect of indole-3-acetic acid is on the curved element (as proposed under (I) and (III)) the size of the stimulation should decrease markedly with increasing glutamic acid concentration (see p. 87). This was not found to be the case. On the whole the graphs obtained seem to accord best with proposal (II). It is possible, however, that the curve may be the early steep part of a hyperbola. If this were so it would assign a relatively high Michaelis constant to the enzyme systems involved. It must be remembered, however, that the ^{14}C

detected in the tissues does not include all the ^{14}C taken up—some has been given off as respiratory CO_2. How the amount of radioactive CO_2 given off is related to external concentration remains to be investigated, and it is possible that correction for this component would modify the experimental curve obtained.

In the case of proposals (I) and (II) it might have been expected that the glutamic acid concentration in the cells would be detectably higher in the auxin-treated samples. But this would not necessarily be the case if, as suggested on p. 9, any glutamic acid entering, either in the presence or absence of auxin, is immediately metabolized. The calculations on pp. 90 and 91 indicated that, at the end of a 2-hour experiment in air, only about 5 per cent. of the tissue could have been in diffusion equilibrium with the external solution. This low figure would be in agreement with the hypothesis that the glutamic acid recovered in the tissues came largely from the cell walls and intercellular spaces. The fact that the specific activity of the glutamic acid recovered was approximately that of the external solution is in accord as well, though it might also be taken to confirm the finding that the native glutamic acid content is very low.

The suppression of the auxin effect in the absence of oxygen suggests that indole-3-acetic acid is exerting its effect on a process which is linked to aerobic metabolism, or which directly involves atmospheric oxygen. Alternatively, it may be the action of auxin itself which is dependent on aerobic metabolism; and here it should be borne in mind that the oxygen requirement may be for the actual uptake of auxin into the region of the cell where the appropriate reaction sites are situated (cf. Reinhold, 1954). This might be the explanation for Cleland and Bonner's (1956) apparently paradoxical result that, while the presence of auxin in the tissue in some form seemed to be necessary during their 'expansion period' (as deduced from experiments with antiauxins), the addition of auxin during this anaerobic period did not promote expansion.

The presence of relatively large amounts of added glutamic acid at the respiratory centres in these experiments (or of derivatives of glutamic acid such as α-ketoglutaric acid) may well have spared endogenous substrate. At any one external concentration of glutamic acid the balance between the amounts of glutamic acid and endogenous substrate respired was altered by the presence of auxin, as shown by the specific activities of the CO_2 evolved (Table IV). One possible explanation for the larger proportion of glutamic acid respired is that auxin directly facilitates uptake of the acid. Alternatively, it may promote the entry of amino-acids into the respiratory cycle.

There is a further possibility. Owing to barriers to diffusion, the concentration of added glutamic acid may be much higher at respiratory centres localized in the cytoplasm adjacent to or interpenetrating the cell wall than at similar centres in the cell interior. If these peripheral centres were those which responded most strongly to the auxin stimulus, a consequence of auxin treatment would be that glutamic acid formed a larger proportion of the substrate respired by the cell as a whole. That the peripheral centres should be

especially sensitive to auxin stimulation is not unreasonable in view of their possible intimate connexion with cell wall growth (cf. Newcomb, 1951).

We wish to express our appreciation of the encouragement given us by Professor G. E. Blackman, Director of this Unit, and by Dr. E. K. Woodford, Assistant Director. We should also like to thank Professor G. E. Briggs, F.R.S., and Professor E. J. Maskell, F.R.S., for helpful discussions of the problem. We are indebted to Miss A. Fisher and Miss P. Clements for technical assistance.

LITERATURE CITED

Akers, T. J., and Fang, S. C. (1956). Studies in plant metabolism. VI. Effect of 2, 4–D on the metabolism of aspartic acid and glutamic acid in the bean plant. *Plant Physiol.* **31**, 34.

Arisz, W. H. (1953). Absorption and transport by the tentacles of *Drosera capensis*. V. Influence on the transport of substances inhibiting enzymatic processes. *Acta Bot. Neer.* **2**, 74.

Barry, J. M. (1956). The use of glutamine and glutamic acid by the mammary gland for casein synthesis. *Biochem. J.* **63**, 669.

Birt, L. M., and Hird, F. J. R. (1956). The uptake of amino-acids by carrot slices. Ibid. **64**, 305.

Blackman, G. E. (1956). Interrelationships between the uptake of 2:4-dichlorophenoxyacetic acid, growth, and ion absorption. In *The chemistry and mode of action of plant growth substances*. Ed. R. L. Wain and F. Wightman. Butterworths Scientific Publications, London.

Bonner, J., Bandurski, R. S., and Millerd, A. (1953). Linkage of respiration to auxin-induced water uptake. *Plant Physiol.* **6**, 511.

Christensen, H. N., Riggs, T. R., and Coyne, B. A. (1954). Effects of pyridoxal and indoleacetate on cell uptake of amino-acids and potassium. *J. Biol. Chem.* **209**, 413.

Cleland, R., and Bonner, J. (1956). The residual effects of auxin on the cell wall. *Plant Physiol.* **31**, 350.

Commoner, B., and Mazia, D. (1942). The mechanism of auxin action. Ibid. **17**, 682.

Hanson, J. B., and Bonner, J. (1954). The relationship between salt and water uptake in Jerusalem artichoke tuber tissue. *Amer. J. Bot.* **41**, 702.

Higinbotham, N., Latimer, H., and Eppley, R. (1953). Stimulation of rubidium absorption by auxins. *Science*, **118**, 243.

Loughman, B. C., and Martin, R. P. (1957). Methods and equipment for the study of the incorporation of phosphorus by intact barley plants in experiments of short duration. *J. Exp. Bot.* **8**, 272.

Maskell, E. J. (1928). Experimental researches on vegetable assimilation and respiration. XVIII. The relation between stomatal opening and assimilation—A critical study of assimilation rates and porometer rates in leaves of cherry laurel. *Proc. Roy. Soc. B.* **102**, 488.

Moore, S., and Stein, W. H. (1948). Photometric ninhydrin method for use in the chromatography of amino-acids. *J. Biol. Chem.* **176**, 367.

—— —— (1951). Chromatography of amino-acids on sulfonated polystyrene resins. Ibid. **192**, 663.

Newcomb, E. H. (1951). Effect of auxin on ascorbic oxidase activity in tobacco pith cells. *Proc. Soc. exp. biol. Med.* **76**, 504.

Ordin, L., Applewhite, T. H., and Bonner, J. (1956). Auxin-induced water uptake by Avena coleoptile sections. *Plant Physiol.* **31**, 44.

Reinhold, L. (1954). The uptake of indole-3-acetic acid by pea epicotyl segments and carrot disks. *New Phytol.* **53**, 217.

—— and Powell, R. G. (1956). A stimulatory action of indole-3-acetic acid on the uptake of amino-acids by plant cells. *Nature Lond.* **177**, 658.

Said, H., and Naguib, M. I. (1952). The effect of heteroauxin on the absorption and assimilation of sucrose by carrot root slices. *Proc. Egypt. Acad. Sci.* **8**, 75.

Van Slyke, D. D., Steele, R., and Plazin, J. (1951). Determination of total carbon and its radioactivity. *J. Biol. Chem.* **192**, 769.

Webster, G. C. (1954). Incorporation of radioactive glutamic acid into the proteins of higher plants. *Plant Physiol.* **29**, 382.

The Effects of Deficiencies of Zinc and Iron on Some Enzyme Systems in *Neurospora*

D. J. D. NICHOLAS AND TESSA GOODMAN

A.R.C. Unit of Plant Nutrition (Micronutrients), Long Ashton Research Station, University of Bristol

Received 22 July 1957

SUMMARY

The effects of the intensity of iron and zinc deficiencies respectively on a range of enzymes in *Neurospora* have been determined. Iron deficiency reduced catalase, peroxidase, cytochrome c reductase, and oxidase (in that order) and DPN'ase activity whereas Zn-deficiency reduced glutamic dehydrogenase. TPNH and DPNH diaphorases were depleted by Zn or Fe deficiency and these enzymes were reconstituted within 24 hours of returning the deficient metal *in vivo* to the felts. Responses of other enzymes to a shortage of either metal varied greatly depending on the degree of the deficiency.

INTRODUCTION

It is well established that trace metals, viz. iron, copper, zinc, manganese, and molybdenum are essential for the growth of fungi (Steinberg, 1939; Mulder, 1939; Nicholas and Fielding, 1951; Nicholas, 1952). It has been shown that deficiencies of trace metals markedly derange enzyme patterns in micro-organisms. Thus Nason, Kaplan, and Colowick (1951) showed that alcohol dehydrogenase and tryptophan synthesizing enzyme was decreased in Zn-deficient *Neurospora* whereas diphosphopyridine nucleotidease was increased. Molybdenum which is an essential constituent of nitrate reductase in bacteria, fungi, and green plants (Nicholas, Nason, and McElroy, 1954; Nicholas and Nason, 1954*a*, *b*, 1955*a*, *b*) was shown to undergo a one-electron change involving valency 6^+ and 5^+ during enzyme action (Nicholas and Stevens, 1955*c*). More recently it was shown that both nitrite and hyponitrite reductases contain haem-Fe and Cu and that hydroxylamine reductase is Mn-dependent (Medina and Nicholas, 1957*a*, *b*). Other effects of trace-metal deficiencies in *Neurospora* include a reduction in catalase and peroxidase activity in Mo-deficient felts (Nicholas, 1956*a*) and depression in hexokinase activity when Zn was limiting (Medina and Nicholas, 1957*a*). The role of Fe and Mo in alternative pathways of electron transfer in a riboflavin-requiring mutant of *Neurospora* has been determined (Nicholas, 1956*b*).

In the present investigation the effects of deficiencies of Zn and Fe on a number of enzymes are described.

METHODS

Fungus. Neurospora crassa, macroconidial, Wild type 5297*a* was kindly supplied by Dr. William D. McElroy, McCollum-Pratt Institute, Johns Hopkins University, Baltimore, U.S.A.

Culture medium. The fungus was grown in the following medium: sucrose, 20 g.; NH_4NO_3, 2 g.; Na tartrate 1 g.; KH_2PO_4, 3 g.; $MgSO_4.7H_2O$, 0·5 g.; NaCl, 0·1 g.; $CaCl_2$, 0·1 g.; biotin, 5 μg., $FeCl_3.6H_2O$, $9{\cdot}6\times10^{-4}$ g.; $ZnSO_4.7H_2O$, $8{\cdot}8\times10^{-3}$ g.; $CuCl_2.2H_2O$, $2{\cdot}7\times10^{-4}$ g.; $MnCl_2.4H_2O$, $7{\cdot}2\times10^{-5}$ g.; $Na_2MoO_4.2H_2O$, $1{\cdot}2\times10^{-5}$ g.; glass-distilled water, 1 litre.

Removal of iron and zinc from the basal medium. The two metals were removed from a solution of the macronutrients, contained in minimal volume of water at pH 5·5 by the 8-hydroxyquinoline-chloroform method (Nicholas, 1952). The trace metals used in the medium were spectrographically pure as supplied by Johnson and Matthey (Hatton Garden, London, E.C. 1). Biotin obtained from the Ashe Laboratories (Leatherhead, Surrey) was dispensed in glass-distilled water.

The culture solution (100 ml.) was put in 500 ml. Erlenmeyer flasks and after sterilizing at 10 lb./sq. in. for 15 min. and cooling, these were inoculated with a spore suspension of the fungus in glass-distilled water. The fungus was grown in the dark at 30° C. and the flasks were shaken periodically to prevent sporulation. The fungus was maintained on nutrient agar slopes and was subcultured weekly.

Preparation of cell-free extracts. The mycelial mats collected in a sintered Büchner funnel were washed well in glass-distilled water and were frozen for 3 hr. or more at −17°. These were then homogenized in three times their weight of cold 0·1M phosphate buffer (pH 7·5) in a mortar and then in a Ten-Broeck glass macerater (made to our specification by Jencons Ltd., Hemel Hempstead, Herts.) at 0°. Tris(hydroxymethyl)amino-methane buffer 0·1M (pH 7·5) was used to extract the 'phosphate' enzymes. The homogenate was centrifuged at 3,000 *g* for 10 min. at 4° C.

Cofactors and other compounds. Triphosphopyridinenucleotide (TPN) 98 per cent. purity, diphosphopyridinenucleotide (DPN) 95 per cent. purity, and cytochrome c were obtained from Sigma Chemical Company, U.S.A., and riboflavin-5-phosphate (FMN) and flavin adenine dinucleotide (FAD) from Nutritional Biochemicals Corporation, U.S.A. DPN was reduced enzymically with crystalline alcohol dehydrogenase, prepared from Acorn yeast (Whittick & Son Ltd., Sheffield 3), by the method of Pullman, Colowick, and Kaplan (1954). TPNH was prepared by using the isocitric dehydrogenase enzyme obtained from acetone powder of pig heart (Ochoa, 1956).

Enzymes assayed

Catalase activity was measured at 37° by the perborate method of Feinstein (1949). The reaction mixture contained 8 ml. 1·5 per cent. (w/v) $NaBO_3.4H_2O$ (pH 6·8); 1·5 ml. 0·1M phosphate buffer (pH 6·8); 0·1 ml. enzyme extract; and was incubated at 37° for 5 min. Then 10 ml. 2N H_2SO_4 was

added and the solution titrated with 0·05N $KMnO_4$. The values are expressed as mmole perborate degraded in 5 min./mg. protein.

Peroxidase was determined by the spectrophotometric method of Smith, Robinson, and Stotz (1949). The reaction mixture contained 1·3 ml. of McIlvaine's buffer (pH 6·0); 1·0 ml. 10^{-3}M reduced 2:6-dichloroindophenol; 0·5 ml. 0·1M H_2O_2; 0·1 ml. enzyme extract. The oxidation of the dye was followed at 625 mμ at 15 sec. intervals. The unit of activity is the change in $\log I_0/I$ of 0·001/min. calculated between 15 and 75 sec./mg. protein.

Cytochrome c oxidase was measured in homogenates of mycelial felts by the method of Smith and Stotz (1949). The reaction solution contained 1·5 ml. 0·2M phosphate (pH 6·8); 1·0 ml. 10^{-3}M reduced 2:6-dichloroindophenol; 0·5 ml. 1×10^{-4}M cytochrome c; 0·2 ml. enzyme extract. The units of activity corrected for endogenous rate are similar to those for peroxidase.

TPN-cytochrome c reductase was measured at 551 mμ. The reaction mixture contained: 2·5 ml. 0·1M phosphate buffer (pH 7·5); 0·5 ml. oxidized cytochrome c, 3×10^{-4}M; 0·1 ml. TPNH (2 μmole/ml.) 0·1 ml. enzyme extract.

DPNH and TPNH oxidases ('diaphorase' systems) were measured by following dye reduction at 625 mμ. The reaction mixture used was as follows: 2·5 ml. 0·1M phosphate (pH 7·5); 0·3 ml. 1×10^{-3}M 2:6-dichloroindophenol; 0·1 ml. 0·1M KCN; 0·1 ml. DPNH (10 mg./ml.) or TPNH (2 μmole/ml.); 0·1 ml. enzyme extract.

Phosphorylase was measured by the method of Whelan (1955). The reaction mixture contained 0·2 ml. 5 per cent. (w/v) soluble starch; 0·5 ml. 0·5M citric acid-NaOH buffer (pH 6·0); 0·1 ml. enzyme extract; 1·7 ml. water equilibrated at 35° for 10 min. and then 1 ml. 0·1M glucose-1-phosphate added. The reaction was stopped after 10 min. by adding 5 ml. 5 per cent. (w/v) trichloroacetic acid and after centrifuging at 3,000 *g.* for 10 min. a sample was taken for P determination. Control tubes were incubated with acid added at the beginning of the reaction. The activity is expressed in μmole P formed/mg. protein.

Phosphoglucomutase was determined by the method of Najjar (1955). The reaction mixture contained 0·1 ml. 0·1M $MgSO_4$; 0·1M glucose-1-phosphate, and 0·1 ml. 0·1M cysteine hydrochloride (pH 7·5) freshly prepared, which was equilibrated at 30° before adding 0·2 ml. enzyme extract. The solution was incubated for 10 min. and then 1 ml. 5N H_2SO_4 added and the volume made to 5 ml. with distilled water. The reaction tubes were put in a boiling water bath to hydrolyse the remaining glucose-1-P. The control tubes were treated with H_2SO_4 as above at zero time. P was determined in 1 ml. of the reaction mixture. The activity is expressed in μmole P/mg. protein or μmole glucose-6-P formed/mg. protein.

Glutamic dehydrogenase was assayed by the method of Olson and Anfinsen (1952). The reaction mixture contained 2·45 ml. 0·1M phosphate buffer (pH 8); 0·1 ml. 0·1M $(NH_4)_2SO_4$; 0·2 ml. TPNH (2 μM./ml.); 0·05 ml. 0·1M and ketoglutarate. The oxidation of the TPNH was followed at 340 mμ.

The unit of activity is the change in log I_0/I_1 of 0·0001/min. calculated between 15 and 75 sec./mg. protein.

Succinic dehydrogenase was determined anaerobically using the method of Burris (1949) in special Thunberg tubes fitted with silica cuvettes. The reaction mixture contained 2·55 ml. 0·1M phosphate buffer (pH 6·8), 0·15 ml. 0·05 per cent. 2:6-dichlorophenolindophenol dye, 0·2 ml. enzyme extract, and 0·1 ml. 0·4M sodium succinate in the sidearm. The Thunberg tube was evacuated and after measuring the endogenous rate of dye reduction at 625 mμ for 1 min. the succinate was added from the sidearm and dye reduction followed at 45-sec. intervals over a 2-min. period. Unit of activity as for glutamic dehydrogenase.

DPN'ase was determined anaerobically by the method of Nason *et al.* (1951). The assay mixture contained 0·1 ml. 10^{-3}M DPN; 0·1 ml. enzyme; 1·3 ml. 0·1M phosphate (pH 7·5) which was incubated for 8 min. at 37° C. 1 ml. 0·5M KCN was added to the control tubes at zero time and to the others after the incubation period. The DPN cyanide addition compound was measured at 325 mμ.

Protein was determined by the Folin's method, Lowry *et al.* (1951).

RESULTS

Effects of iron and zinc deficiencies on enzymes

Iron enzymes. The incidence of Fe or Zn deficiencies on a range of enzymes are illustrated in Figs. 1 and 2. As Zn-deficiency became more severe, cytochrome c oxidase was decreased whereas its reductase remained unchanged. Catalase and, in particular, peroxidase were increased in activity when the felt weight was reduced to 71 per cent. and 47 per cent. of normal values but peroxidase activity returned to normal when the deficiency was most severe.

As was to be expected the more intense the iron deficiency the more reduced were the activities of the iron enzymes in extracts of the felts. Catalase was the first enzyme to be affected by iron deficiency, then peroxidase, followed by cytochrome c reductase and its oxidase.

Dehydrogenase enzymes. TPNH and DPNH 'diaphorases' were both depressed in activity as Zn-deficiency became more severe.

Apart from a stimulation in enzyme activity in C (47 per cent. by weight of control), succinic dehydrogenase was unchanged. Glutamic acid dehydrogenase was markedly reduced by Zn-deficiency.

Both diaphorases were reduced whereas glutamic dehydrogenase was increased by a drastic Fe deficiency (D).

Phosphate enzymes and DPN'ase. A marked increase in DPN'ase activity was found when Zn was deficient especially in C. Phosphoglucomutase although reduced in felts from treatments B and C, was increased in D when Zn deficiency was most severe. Phosphorylase activity increased at all levels of Zn-deficiency.

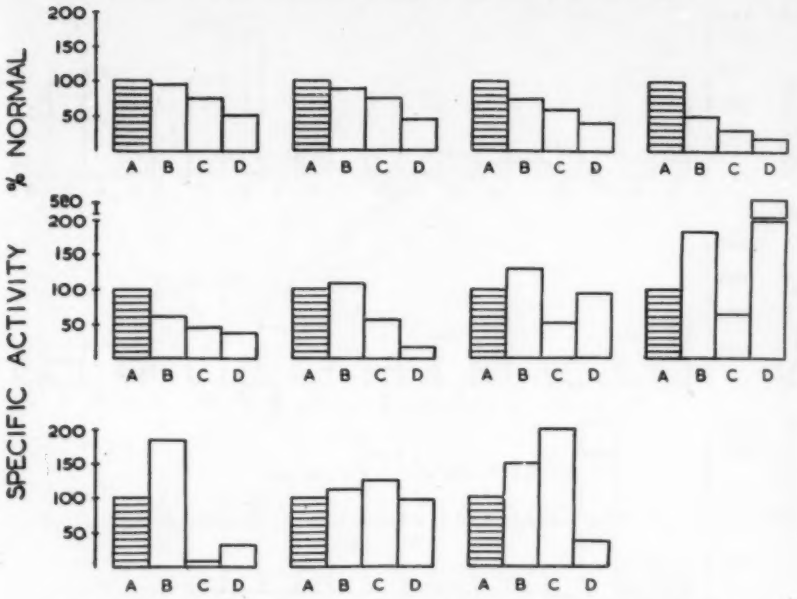


FIG. 1. The effect of the intensity of Fe-deficiency on some enzymes in *Neurospora*.

The intensity of deficiency is measured by the dry weights of mycelial felts. A refers to normal felts; B, deficient felt wt. 79% normal; C, felt wt. 56% normal; D, felt wt. 19% normal.

Top row: Cytochrome c oxidase, cytochrome c reductase, peroxidase, catalase.
Middle row: TPNH diaphorase, DPNH diaphorase, succinic dehydrogenase, glutamic dehydrogenase.
Bottom row: DPN'ase, phosphoglucomutase, phosphorylase.

A drastic deficiency of iron (C and D) depressed DPN'ase activity but had little effect on succinic dehydrogenase. Phosphorylase was stimulated at the first two deficiency levels B and C but was drastically reduced in D when Fe deficiency was more pronounced.

DPNH and TPNH 'diaphorases'

Effect of metal deficiencies. The effects of individual deficiencies of metals on DPNH and TPNH diaphorases are shown in Table I. It is clear that a deficiency of either Fe or Zn drastically depressed the activities of both enzymes.

Effects of graded amounts of iron and zinc. The effect of increasing the Fe content of the basal medium from deficiency to sufficiency levels on the two enzymes in the felts is illustrated in Fig. 3. The activity of both enzymes was increased from 0 to 15 μg. Fe/100 ml. medium.

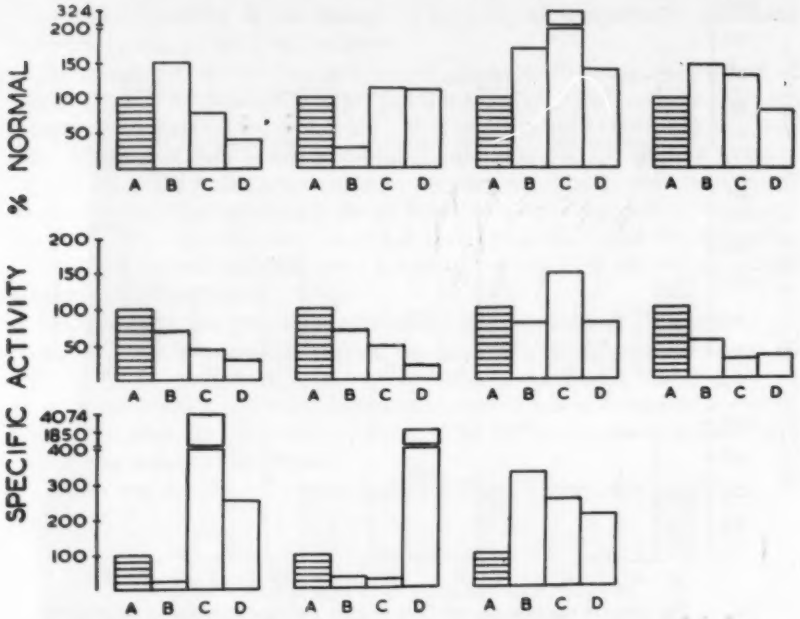


FIG. 2. The effect of the intensity of Zn-deficiency on some enzymes in *Neurospora*.

The intensity of deficiency measured by dry weights of mycelial felts. A, normal felts; B, dry wt. 71% normal; C, dry wt. 47% normal; D, dry wt. 35%.

Top row: Cytochrome c oxidase, cytochrome c reductase, peroxidase, catalase.
Middle row: TPNH diaphorase, DPNH diaphorase, succinic dehydrogenase, glutamic dehydrogenase.
Bottom row: DPN'ase, phosphoglucomutase, phosphorylase.

TABLE I

Effect of metal deficiencies on DPNH and TPNH 'diaphorases'

(Specific activities of the two enzymes and dry weights as % of normal values)

	Control	omit Fe	omit Cu	omit Zn	omit Mn	omit Mo
DPNH	100	33	89	42	76	96
'diaphorase'	100	17	94	37	83	97
TPNH	100	27	92	31	79	92
'diaphorase'	100	30	86	23	72	98
Dry Weight	100	50	60	43	50	60
	100	20	40	25	29	42

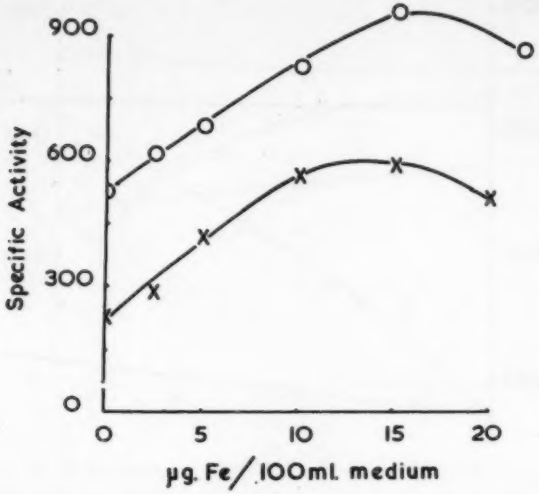


FIG. 3. The effect of adding graded amounts of Fe to the medium on the diaphorases in the felts.

○——○ TPNH diaphorase
×——× DPNH diaphorase

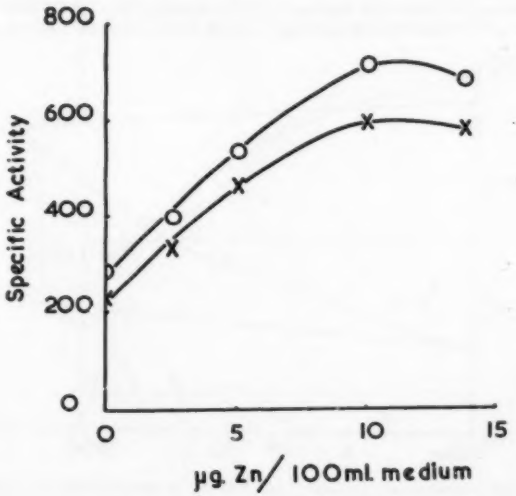


FIG. 4. The effect of adding graded amounts of Zn to the medium on the two diaphorases in the felts.

○——○ TPNH diaphorase
×——× DPNH diaphorase

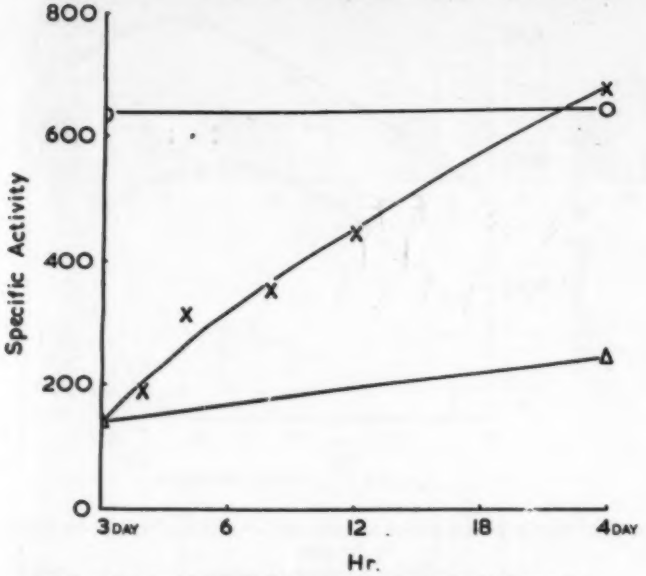


FIG. 5. Reconstitution of DPNH diaphorase in Fe-deficient felts.

O——O enzyme from felts grown in complete medium;
Δ——Δ enzyme from Fe-deficient felts;
×——× 200 μg Fe/litre added aseptically to Fe-deficient felts after 3 days' growth and the enzyme production measured at intervals within a further 24-hr. incubation period.

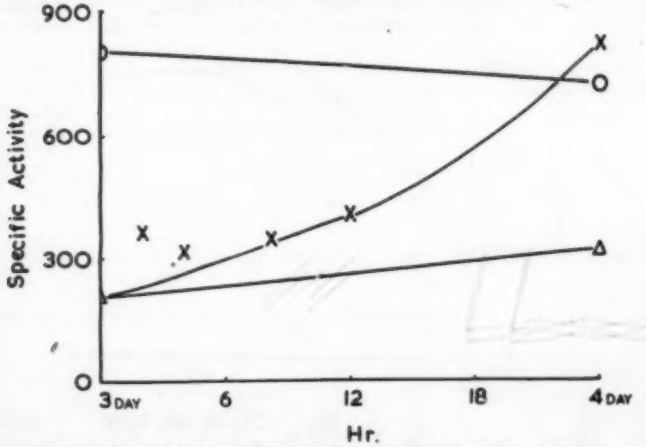


FIG. 6. Reconstitution of TPNH diaphorase in Fe-deficient felts.

O——O enzyme from felts grown in complete medium;
Δ——Δ enzyme from Fe-deficient felts;
×——× 200 μg Fe/litre added aseptically to Fe-deficient felts after 3 days' growth and the enzyme production measured at intervals within a further 24-hr. incubation period.

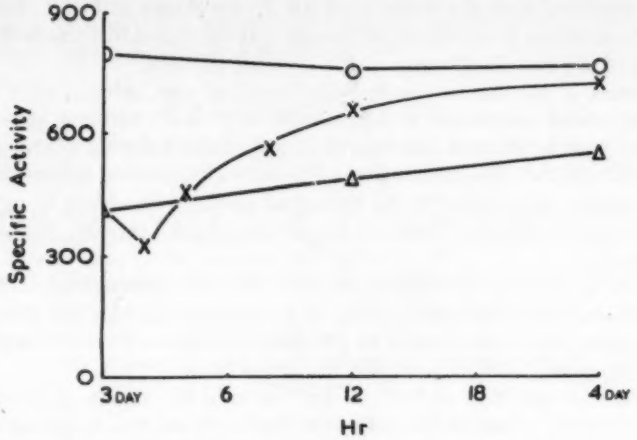


FIG. 7. Reconstitution of DPNH diaphorase in Zn-deficient felts.

○——○ enzyme from felts grown in complete medium;
△——△ enzyme from Zn-deficient felts;
×——× 200 μg Zn/litre added aseptically to Zn-deficient felts after 3 days' growth and the enzyme production measured at intervals within a further 24-hr. incubation period.

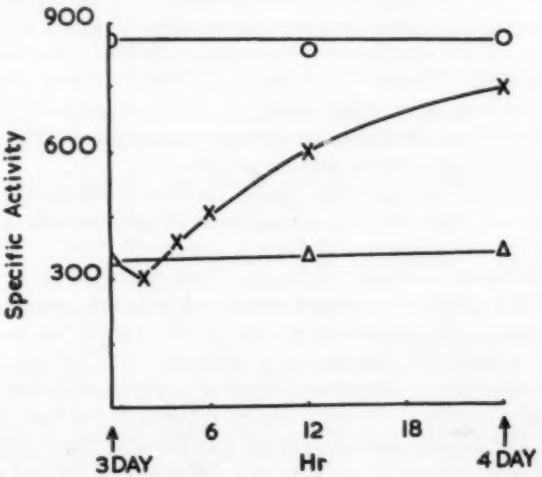


FIG. 8. Reconstitution of TPNH diaphorase in Zn-deficient felts.

○——○ enzyme from felts grown in complete medium;
△——△ enzyme from Zn-deficient felts;
×——× 200 μg Zn/litre added aseptically to Zn-deficient felts after 3 days' growth and the enzyme production measured at intervals within a further 24-hr. incubation period.

The results of a similar experiment for Zn are shown in Fig. 4. Increasing Zn in the medium from 0 to 20 μg/100 ml. was paralleled by increased specific activity of the two diaphorases in the mycelial extracts.

Induction of the diaphorases by added iron and zinc. After 3 days' growth iron was added aseptically to normal and to omit-Fe cultures and the two enzymes were determined in felts collected at intervals during a further 24–hr. incubation period. The results are illustrated in Figs. 5 and 6. It is clear that both enzymes were reconstituted to normal amounts by adding 15 μg Fe/100 ml. medium. Addition of Fe in *vivo* to normal felts, however, did not affect the enzyme.

Results of similar experiments in which Zn was returned to normal and Zn-deficient felts, illustrated in Figs. 7 and 8, show that DPNH and TPNH diaphorases were reconstituted in the deficient felts to about 90 and 45 per cent. respectively of their activities in normal felts.

Neither the addition of Fe^{2+} or Fe^{3+} *in vitro* to extracts of Fe-deficient felts nor Zn to those of Zn-deficient felts activated the diaphorases. The addition of boiled extract of normal felts to Fe-deficient felts reactivated the DPNH system by 36 per cent. and the TPNH diaphorase by 31 per cent. as did the addition of FAD.

DISCUSSION

When a metal is essential for enzyme production it is usual to find that its activity is considerably reduced in tissues deficient in the nutrient. The results given herein show that for some enzymes the intensity of either Fe or Zn deficiency did not progressively increase or decrease their activities but had a variable effect. Thus it is only when an enzyme shows a proportionality between the amount of nutrient added to the medium from deficiency to sufficiency levels, and its increased activity in the tissues that it is safe to conclude that it is required for enzyme activity.

As iron deficiency became more severe, catalase, peroxidase and the cytochrome c enzymes were reduced in that order. This may reflect the relative affinity of Fe for the porphyrin of these enzymes.

DPNH-diaphorase obtained from pig-heart particles is a flavoprotein containing FAD and its Fe content was found to be one-twentieth of cytochrome c reductase of comparable purity. Mahler (1956) has suggested that diaphorase is a degraded cytochrome c reductase in which Fe has been removed. The experiments described herein show, however, that a deficiency of Fe markedly impeded dye reduction in extracts of the felts, so that both TPNH and DPNH diaphorases are in fact Fe-dependent. The two diaphorases are also reduced in activity by a deficiency of Zn but whether the metal is a constituent of the diaphorase systems or whether its effect is on the elaboration of the protein moieties of the enzymes, remains to be determined.

It is of interest that glutamic dehydrogenase recently shown to contain Zn in animal tissues, Vallee *et al.* (1955) is markedly reduced in Zn-deficient

felts. The increased DPN'ase activity with Zn deficiency first observed by Nason *et al.* (1951) is also confirmed here.

LITERATURE CITED

Bessey, O. A., Lowry, O. H., and Brock, M. J. (1946). A method for the rapid determination of alkaline phosphatase with five cubic millimetres of serum. *J. Biol. Chem.* **164**, 321.

Burris, R. H. (1949). Thunberg techniques for estimation of dehydrogenase activity, in *Manometric Techniques and Tissue Metabolism*, Eds. Umbreit, Burris, and Staufer. Burgess Publishing Co. Minneapolis, Min. U.S.A., p. 105.

Feinstein, R. N. (1949). Perborate as substrate in a new assay of catalase. *J. Biol. Chem.* **180**, 1197.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with Folin phenol reagents. Ibid. **193**, 275.

Mahler, H. (1956). Nature and function of flavoproteins. *Adv. in Enzym.* **17**, 233.

Medina, A., and Nicholas, D. J. D. (1957*a*). Zinc-dependent hexokinase from *Neurospora crassa*. *Nature* **179**, 87.

—— —— (1957*b*). Metallo-enzymes in the reduction of nitrite to ammonia in *Neurospora*. *Biochim. Biophys. Acta* **25**, 138.

Mulder, E. G. (1939). On the use of micro-organisms in measuring a deficiency of copper, magnesium and molybdenum in soils. *Antonie van Leeuwenhoek*, **6**, 99.

Nason, A., Kaplan, N. O., and Colowick, S. P. (1951). Changes in enzymatic constitution of zinc-deficient *Neurospora*. *J. Biol. Chem.* **188**, 397.

Najjar, V. A. (1955). Phosphoglucomutase from muscle, in *Methods in Enzymology*, Eds. Colowick, S. P., and Kaplan, N. O. New York, Academic Press: Vol. 1, p. 294.

Nicholas, D. J. D., and Fielding, A. H. (1951). The use of *Aspergillus niger* (M) for the determination of magnesium, zinc, copper and molybdenum available in soils to crop plants. *J. Hort. Sci.* **24**, 125.

—— (1952). The use of fungi for determining trace metals in biological materials. *Analyst* **77**, 629.

—— Nason, A., and McElroy, W. D. (1954). Molybdenum and nitrate reductase. *J. Biol. Chem.* **207**, 341.

—— —— (1954*a*). Molybdenum as a constituent of nitrate reductase. Ibid. **207**, 353.

—— —— (1954*b*). Mechanism of action of nitrate reductase from *Neurospora*. Ibid. **211**, 183.

—— —— (1955*a*). Diphosphopyridine nucleotide-nitrate reductase from *Escherischia Coli*. *J. Bact.* **69**, 580.

—— —— (1955*b*). Role of molybdenum as a constituent of nitrate reductase from soybean leaves. *Plant Physiol.* **30**, 135.

—— and Stevens, H. M. (1955*c*). Valency changes of molybdenum during the enzymatic reduction of nitrate in *Neurospora*. *Nature* **176**, 1066.

—— (1956*a*). Effect of molybdenum deficiency on catalase and peroxidase in *Neurospora*. Ibid. **178**, 148.

—— (1956*b*). Trace metal requirements and some enzyme systems in a riboflavin requiring mutant of *Neurospora crassa*. *J. Gen. Microbiol.* **15**, 470.

—— (1957). Role of metals in enzymes with special reference to flavoproteins. *Nature* **179**, 800.

Ochoa, S. (1956). Isocitric dehydrogenase system from pigheart, in *Methods in Enzymology*, Eds. Colowick, S. P., and Kaplan, N. O. New York, Academic Press: Vol. 1, p. 699.

Olson, J. A., and Anfinsen, C. B. (1952). The crystallization and characterization of l-glutamic acid dehydrogenase. *J. Biol. Chem.* **197**, 67.

Pullman, M., Colowick, S. P., and Kaplan, N. O. (1954). Comparison of diphosphopyridine nucleotide with its deaminated derivative in various enzyme systems. Ibid. **135**, 727.

Smith, F. G., Robinson, W. B., and Stotz, E. (1949). A colorimetric method for the determination of peroxidase in plant material. Ibid. **179**, 881.

SMITH, F. G., and STOTZ, E. (1949). A colorimetric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **179**, 891.

STEINBERG, R. A. (1939). Growth of fungi in synthetic nutrient solutions. *Bot. Rev.* **5**, 6, 327.

VALLEE, B. L., ALDESTEIN, S. J., and OLSON, J. A. (1955). Glutamic dehydrogenase of beef liver; a zinc metallo-enzyme. *J. Amer. Chem. Soc.* **77**, 5196.

WHELAN, W. J. (1955). Phosphorylases from plants, in *Methods in Enzymology*, Eds. Colowick, S. P., and Kaplan, N. O. New York, Academic Press: Vol. 1, p. 192.

Microbiological Synthesis of Fat

THE EFFECTS OF NITROGEN LEVEL ON THE METABOLISM OF *PENICILLIUM LILACINUM* THOM IN A SUCROSE MEDIUM

KAISER NAGUIB[1] AND THOMAS KENNEDY WALKER

College of Science and Technology, The University, Manchester

Received 5 July 1957

SUMMARY

1. A study has been made of the relationships between the syntheses of carbohydrate, protein, and fat by *Penicillium lilacinum* Thom in presence of different amounts of sodium nitrate in a defined sucrose salts medium.

2. Under the defined experimental conditions increases in the concentration of NO_3^- in the medium were followed by increases in the rates at which nitrogen and sugar were taken up by the fungus, in the quantities assimilated, and in total and protein nitrogen in the felt. These conditions prevailed so long as unassimilated sugar was available.

3. Media of lower NO_3^- concentration (for example, 0·32 or 0·64 per cent. (w/v) $NaNO_3^-$) yielded felts richer in carbohydrate than were those grown in media of higher NO_3^- content (0·96 or 1·28 per cent. (w/v) $NaNO_3$). The carbohydrate content of the felts increased gradually until the sugar in the medium was exhausted; carbohydrate content then decreased.

4. Media of lower NO_3^- concentration were more conducive to fat synthesis than those of higher NO_3^- content.

INTRODUCTION

THE effects of the concentration of nitrogen and of the N:C ratio in the medium on the production of fat by micro-organisms have been studied by Smedley-Maclean (1922), Prill, Wenck, and Peterson (1935), Heide (1939), Bichkovskaya (1940), Bernhauer and Rauch (1948), and Kleinzeller (1948). In the course of the present inquiry the authors have investigated the fat-producing capacity of *Penicillium lilacinum* Thom in a defined medium in which the quantity of $NaNO_3$ used as source of nitrogen was the only variable. The object was to follow quantitatively the syntheses of protein, carbohydrate, and fat as influenced by the quantity of nitrogen available and to trace during the progress of growth the relationships between these three different components.

EXPERIMENTAL

Strain. Penicillium lilacinum Thom was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

[1] Present address: Botany Department, Faculty of Science, Cairo University, Egypt.

Media and conditions of cultivation. The mould was maintained by subculturing at intervals of 14 days on Dox's agar medium consisting of (g./l.): KH_2PO_4, 1; $MgSO_4$, $7H_2O$, 0·5; KCl, 0·5; $FeSO_4$, $7H_2O$, 0·01; $NaNO_3$, 2; sucrose, 15; agar 15. The mould was then grown as surface cultures on a basal liquid medium of the composition (g./l.): K_2SO_4, 0·11; NaH_2PO_4, $2H_2O$, 7·30; $MgSO_4$, $7H_2O$, 5·00; $ZnSO_4$, $7H_2O$, 0·05; $FeCl_3$, $6H_2O$, 0·16; sucrose, 170; made up with distilled water and the pH value adjusted with NaOH to 6·8. This medium was completed by addition of $NaNO_3$ at the following concentrations (per cent. w/v): 0·32, 0·64, 0·96, 1·28. The medium was dispensed in volumes of 50 ml. in conical flasks of 250 ml. capacity, which had necks of 25 mm. diameter. These were plugged and sterilized at 15 lb. pressure for 10 minutes, cooled and inoculated with a suspension of spores obtained from slope cultures on the Dox's agar medium which had been incubated for 10 days at 25°. The flask cultures were incubated at 25° and at intervals of 4, 6, 8, 10, 12, and 14 days, respectively, five replicate flasks were withdrawn and their contents analysed. The metabolism solution was separated from the mycelial felts and the latter were washed with water and dried in a vacuum oven at 60°, after which they were weighed. They were then ground and aliquot samples were taken for estimation of protein, total carbohydrate, and fat. The filtered metabolism solution was made up to a definite volume and samples were taken for estimation of residual sugar and total nitrogen, respectively.

Estimations of sucrose in the medium and of *total carbohydrate* in the felt were carried out in the manner described by Saïd and Naguib (1955) when reporting similar work with *Fusarium moniliforme*.

Estimations of total nitrogen in the medium and in the felt were performed by micro-Kjeldahl method after treatment of the sample with sulphuric-salicylic acid mixture and hydriodic acid (Strouts, Gilfillan, and Wilson, 1955) in order to include nitrate nitrogen.

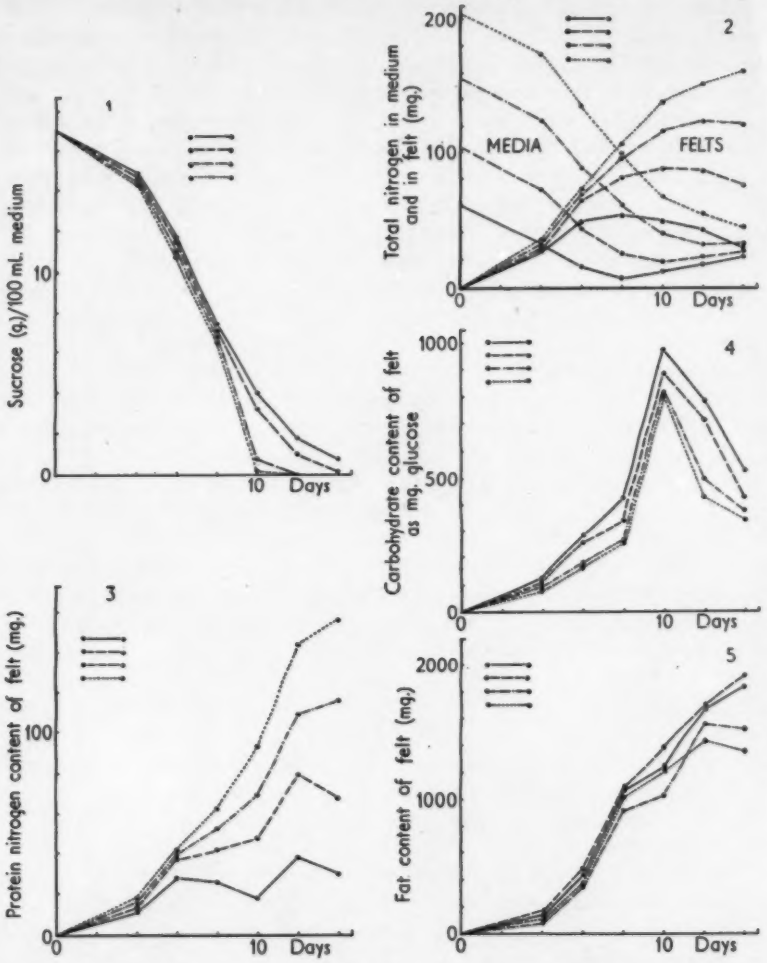
Estimation of protein nitrogen in the felt was undertaken after the soluble nitrogen fraction had been separated from the insoluble protein by trichloroacetic acid. Steam distillation of the ammonia was carried out in the apparatus described by Scandrett (1953).

Estimation of fat in the felt was carried out as described by Woodbine, Gregory, and Walker (1951).

RESULTS

Sugar uptake. From Fig. 1 it can be seen that within the experimental conditions the assimilation of sugar by the mould was accelerated by high initial nitrogen supply in the medium. At the highest nitrogen level the sugar was almost entirely assimilated in 10 days.

Nitrogen uptake. Loss of nitrogen from the different media (Fig. 2) proceeded gradually down to minimum values which differed with the different initial nitrogen concentrations. In the cases of the two media in which the nitrogen concentrations were lower the curves indicate that maximum nitrogen



FIGS. 1–5. Sucrose and nitrogen contents of media and protein, carbohydrate, and fat contents of felts grown on the following percentages of $NaNO_3$: ——— 0·32, — — — 0·64, — · — · 0·96, and - - - - - 1·28.

1. Changes in sucrose content of medium with time.
2. Changes in the total nitrogen content of medium and of mycelial felt with time.
3. Changes in protein nitrogen content of mycelial felt with time.
4. Changes in total carbohydrate content of mycelial felt with time.
5. Changes in fat content of mycelial felt with time.

content of the felt was achieved in about 8 and 10 days, respectively, after which nitrogen began to pass from the felt in the medium. Similar behaviour was noted by Hockenhull with *Penicillium notatum* (1946) and with *Aspergillus*

nidulans (1950). Such loss of nitrogen from the felt is to be ascribed to autolysis. In the cases of the two media initially of higher nitrogen concentration, accumulation of nitrogen in the felt was still proceeding on the fourteenth day, when the experiment was stopped.

Protein nitrogen in the felts. From Fig. 3 it can be seen that in the felts fed with the two higher concentrations of nitrogen protein was built up gradually and steadily during 12 days, after which the rate of synthesis declined. On the other hand, the felts nourished in the media of lower nitrate content accumulated their protein at lower rates, the maximum contents being reached at 12 days, after which the protein content commenced to fall.

Carbohydrate content of the felts. The total carbohydrate content (Fig. 4) increased slightly in all cases during the first 8 days of incubation and then much more rapidly in the period 8–10 days, when maximum values were attained, thereafter it commenced to fall in all cases, but the drop was steeper in the two media of higher nitrogen content. The curves show clearly that high carbohydrate content of the felt was promoted by low concentration of nitrogen in the medium.

Fat content of the felts. Fig. 5 shows that the fat content in all cases started to increase more rapidly after the fourth day of incubation. The maximum amount of fat was produced in the medium which contained 0·64 per cent. (w/v) of $NaNO_3$; however, the medium of lowest nitrogen content accumulated almost as much fat. The two media of higher nitrogen content gave rise to felts in which maximum fat content was attained at 12 days, after which there was a gradual fall in accumulated fat. This fall was not observed in the two former cases.

DISCUSSION

In the present work, higher rates of sugar utilization were observed in the media of higher nitrogen content and resulted in the formation of mycelial felts of relatively lower carbohydrate content. This behaviour was accompanied by a higher rate of nitrogen uptake the sugar absorbed being utilized for building up protein rather than for production of reserve carbohydrate. In these cases protein synthesis was enhanced and continued after the media had been completely depleted of sugar. Raaf (1941), who studied the behaviour of *Endomyces vernalis*, stated that protein synthesis persisted until complete exhaustion of the carbohydrate component of the culture medium, after which breakdown processes commenced in the mycelial constituents. The more detailed data recorded in the course of the present study showed that in the presence of an ample supply of nitrogen, and after exhaustion of the sugar of the medium, synthesis of protein could continue at the expense of the reserve of carbohydrate accumulated in the mycelium, which was observed to be diminishing while nitrogen uptake was still in progress. On the other hand, in the cases of growth in the two media of lower nitrogen level, the quantities of nitrogen in the media had declined to very small amounts at 8 days, while in neither instance was the sucrose supply yet exhausted. However, while

the carbohydrate content of the cells had dropped steeply by the tenth day, synthesis of fat continued until the end of the experimental period, showing that in these instances the formation of fat was being maintained at the expense of reserve carbohydrate. At the same time, proteolysis commenced, as indicated by an increase in the nitrogen content of the medium. Our finding that the employment of media of high nitrogen concentration for cultures of *P. lilacinum* leads to formation of felts of relatively low fat content confirms the experience of earlier workers (Smedley-Maclean, 1922; Prill *et al*, 1935; Bichkovskaya, 1940; Kaibara, 1948; Gregory and Woodbine, 1953; Sheilah Murray and Walker, 1956) with different micro-organisms.

Finally, the fact that the syntheses of protein and of fat in *P. lilacinum* are in an inverse relationship controlled by the N:C ratio in the medium, is in accord with the previous findings of Raaf (1941), in work with *Endomyces vernalis*, of Bernhauer and Rauch (1948), who employed *Mucor circinelloides*, and of Enebo, Anderson, and Lundin (1946). The latter authors studied the behaviour of *Rhodotorula gracilis* and found the optimal N:C ratio for fat synthesis to be 1:80, whereas in our case with *P. lilacinum* it is 1:65.

LITERATURE CITED

Bernhauer, K., and Rauch, J. (1948). Beiträge zur microbiologischen Eiweiß-und Fettsynthese. *Biochem. Z.* **319**, 102–19.

Bichkovskaya, A. L. (1940). The formation of fat by the fungus *Endomyces vernalis*. I. The influence of the composition of the substrate on the formation of fat by *E. vernalis* under conditions of film growth. *Microbiology* (Moscow), **8**, 1170–80. (See *Chem. Abs.* 1942, **42**, 5850.)

Enebo, L., Anderson, L. G., and Lundin, H. (1946). Microbiological fat synthesis by means of *Rhodotorula* yeast. *Arch. Biochem.* **11**, 383–95.

Gregory, Margaret, E., and Woodbine, M. (1953). Microbiological synthesis of fat. The effects of varying pH, temperature, nitrogen, incubation period, and sugar concentration on fat-producing moulds. *J. Exp. Bot.* **4**, 314–18.

Heide, S. (1939). Zur Physiologie und Cytologie der Fettbildung bei *Endomyces vernalis* mit einem Beitrage zur Methodik der quantitativen Bestimmung kleinster Fettmengen. *Arch. Mikrobiol.* **10**, 135–88.

Hockenhull, D. J. D. (1946). Studies in penicillin production by *P. notatum*: I. A preliminary study of the metabolism of carbon, nitrogen, sulphur, and phosphorus. *Biochem. J.* **40**, 337–43.

—— (1950). Studies in the metabolism of mould fungi. I. A preliminary study of the metabolism of carbon, nitrogen, and sulphur by *Aspergillus nidulans*. *J. Exp. Bot.* **1**, 194–200.

Kaibara, T. (1948). Fat production by moulds. I. *J. Agric. Chem. Soc.* Japan. **22**, 89–90.

Kleinzeller, A. (1944). Fat formation in *Torulopsis lipofera*. *Biochem. J.* **38**, 480–92.

—— (1948). Synthesis of lipides. *Adv. Enzymol.* **8**, 299–341.

Murray, Sheilah, and Walker, T. K. (1956). Mycological formation of fat. III. Media conducive to formation of fat from sucrose by *Penicillium soppi* Zaleski in surface culture. *J. Sci. Food. Agric.* **7**, 237–40.

Prill, E. A., Wenck, P. R., and Peterson, W. H. (1935). The chemistry of mould tissue. VI. Factors influencing the amount and nature of fat produced by *Aspergillus fischeri*. *Biochem. J.* **29**, 21–33.

Raaf, H. (1941). Beiträge zur Kenntnis der Fett- und Eiweißsyntheses bei *Endomyces vernalis* und einigen anderen Mikroorganismen. *Arch. Mikrobiol.* **12**, 131–82.

Said, H., and Naguib, K. (1955). Absorption and assimilation of monosaccharides by *Fusarium moniliforme* Sheldon. *Proc. Egypt. Acad. Sci.* **11**, 37–48.

SCANDRETT, F. J. (1953). A modified all-glass apparatus for the determination of nitrogen by the micro-Kjeldahl method. *Analyst.* **78**, 734–7.

SMEDLEY-MACLEAN, I. (1922). The conditions influencing the formation of fat by the yeast cell. *Biochem. J.* **16**, 370–9.

STROUTS, C. R. N., GILFILLAN, J. H., and WILSON, H. N. (1955). *Analytical Chemistry* Vol. I. Oxford University Press, 356.

WOODBINE, M., GREGORY, MARGARET E., and WALKER, T. K. (1951). Microbiological synthesis of fat. Preliminary survey of the fat-producing moulds. *J. Exp. Bot.* **2**, 204–11.

The Interaction of Cations in Absorption by Plant Tissues

L. J. MIDDLETON[1] AND R. SCOTT RUSSELL[1]

Department of Agriculture, University of Oxford

Received 7 September 1957

INTRODUCTION

WHEN the roots of whole plants or excised plant material are placed into salt solutions two apparently distinct steps in the absorption process can be recognized (Broyer, 1951; Steward and Millar, 1954; Russell, 1954; Epstein, 1956). A rapid initial absorption is followed by a slower long-continued second phase; only the latter is closely dependent on concurrent metabolism.

Observation of the initial phase depends to some extent on the method of measurement. It is not always obvious when conductivity methods are employed (Lundegårdh, 1949) though it may be observed (Robertson, Wilkins, and Weeks, 1951). It is very pronounced, however, when the absorption of ions not normally present in the tissue in high concentration, for example, manganese (Stiles and Dent, 1946) or rubidium (Steward and Harrison, 1939) is measured. Clear evidence of the rapid initial absorption is also obtained when uptake is measured by means of radioactive isotopes, since their use makes it possible to measure the exchangeability of a previously absorbed ion. Moreover, absorption periods of minutes or even seconds can be used and the initial step of entry can be studied without complications due to ions entering further processes.

In the experiments described in this paper the entry of cations into carrot disks has been studied for periods ranging from 5 minutes to 24 hours. Previous investigations in this laboratory indicated the importance of competition between ions in the initial phase of entry (Russell and Ayland, 1955) and further information on this question was sought in experiments of short duration. In addition the fate of previously absorbed ions was examined by measurement of exchangeability. Experiments of longer duration (i.e. 24 hours) were undertaken to provide information on the relationship of the mechanisms responsible for the transport of ions from the external solutions to the interior of the cell.

EXPERIMENTAL TECHNIQUE

Tissue. Disks, 1 cm. in diameter and 1 mm. thick, consisting mainly of ploem parenchyma were cut from carrot roots. These were washed in demineralized water for 7 days before use.

[1] Present address: A.R.C. Radiobiological Laboratory, Grove, Wantage, Berks.

Method. Boiling-tubes 8 × 1½ in. were used as experimental vessels. Twenty disks, weighing about 2 g. and 50 ml. of demineralized water were placed in each tube and a stream of air was passed sufficiently rapidly to circulate the disks. A duplicate set of tubes contained 50 ml. of the experimental solution at twice the desired final concentration. All tubes were equilibrated at 25° C. After equilibration the disks and demineralized water were tipped into the experimental solutions and harvested after a predetermined period. A small part of the solution was removed for analysis and the rest, together with the disks, was poured on to a sintered plate to which suction was applied. This removed the solution from the disks very rapidly and dried the disk surface of excess liquid in less than 1 minute.

Disks and excised roots were transferred to small corked tubes containing 13 ml. of a solution with a high concentration of the ion being studied (potassium was substituted for rubidium because of the high cost of rubidium salts) and N/10 nitric acid. The tissue was killed by this treatment and after standing overnight to allow equilibration of the absorbed ions with the solution, 10 ml. of the solution was withdrawn for assay. This method proved simple and sensitive; replicates agreed to within a few percent.

Analysis. Uptake was measured using radioactive isotopes rubidium–86 and strontium–89 of the elements being studied. These isotopes were chosen because of their convenient half-lives and β-rays which are counted efficiently in solution by simple techniques. Isotopes of potassium and calcium are considerably less convenient. In addition the metabolism of strontium is being extensively studied in this laboratory and elsewhere because of its importance as a constituent of fall-out from nuclear weapons. Assay procedures were similar to those described by Martin and Russell (1950). The percentage uptake of the radioactivity was determined and from this the total uptake of the non-radioactive isotope was calculated. Results are expressed as microequivalents per gram fresh weight (μeq./g.f.wt.). In some cases uptake is expressed as a percentage of one treatment where comparison is facilitated by this method.

Rubidium–86 was obtained from irradiation of spectrographically pure rubidium chloride and strontium–89 was obtained carrier free as a separated fission product. The concentration of the radioactive isotopes was 10–15 μc./l.

RESULTS

Interaction amongst cations. Russell and Ayland (1955) examined the interaction of cations during absorption by carrot disks in an attempt to distinguish between absorption by an exchange mechanism and by a mass diffusion of solute. They argued that if diffusion were the rate limiting process the entry of one cation should not be affected by the presence of another. On the other hand, competition was to be expected if entry were controlled by exchange. Hence the finding of competition for absorption between pairs of univalent ions was taken as evidence of an exchange mechanism. These experiments were repeated and extended to include a wider range of ions.

The effect of some univalent ions on the absorption of rubidium and of bivalent cations on the absorption of strontium is shown in Table I. In each case the competing ion was at the same concentration as the measured ion (0·4 meq./l.). In one treatment extra rubidium or extra strontium was added to compare the effect of added non-radioactive isotope with that of adding different elements; this extra carrier was ignored in calculating total absorption, to facilitate comparison between treatments. It can be seen that the absorption of rubidium or strontium was reduced in the presence of added cations. The reduction was greatest with ions of high atomic number (caesium, barium) smallest with ions of low atomic number (lithium, magnesium). The effect of the addition of potassium to rubidium solutions or of calcium to strontium was not significantly different from the addition of an equivalent amount of an inactive isotope of the element measured.

TABLE I

The effect of cations on the uptake (as μeq./g.) of rubidium or strontium in 5 mins. by carrot disks. All ions 0·4 meq./l.

Rubidium

Competing ion	Rb uptake	% reduction
None	2·48	—
Lithium	1·88	24·2
Sodium	1·67	32·7
Potassium	1·51	39·2
Rubidium	1·48	40·3
Caesium	1·41	43·1
Sig. diff.	0·09	—

Strontium

Competing ion	Sr uptake	% reduction
None	3·28	—
Magnesium	2·35	28·4
Calcium	2·16	34·1
Strontium	2·17	33·9
Barium	1·99	39·3
Sig. diff.	0·07	—

It is to be expected that differences in valency would influence competitive power to an even greater extent than differences in atomic size amongst elements of the same valency. An experiment was carried out in which the uptake of rubidium was measured in the presence of a range of concentrations of strontium and the uptake of strontium in the range of concentrations of rubidium. It is clear from Fig. 1 that strontium caused a very great reduction in the uptake of rubidium; the effect of rubidium on the uptake of strontium was considerably less.

Fate of absorbed ions. Experiments were carried out to determine the extent to which strontium and rubidium absorbed in 5 minutes from 0·4 meq./l. or 0·2 meq./l. solution respectively are lost when carrot disks are placed in

demineralized water or carrier solutions of the following concentration, 0·4, 3·2, and 12·8 meq./l. for 5, 20, and 90 minutes. The results are presented in Figs. 2 and 3. The striking feature of Fig. 3 is that no strontium was released into demineralized water even after 90 minutes' washing but that 90 per cent. was lost into the highest concentration of strontium chloride. In contrast

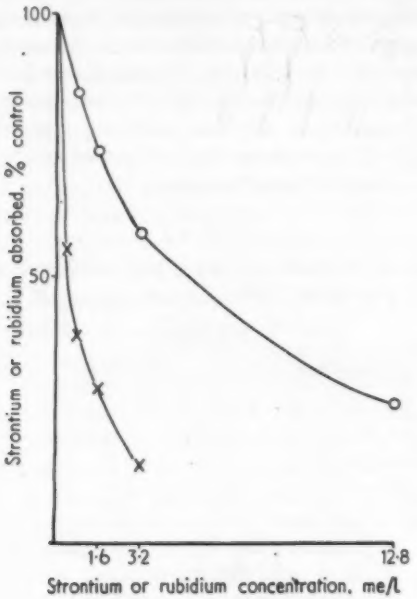


FIG. 1. The effect of rubidium on the absorption of strontium (○——○) and of strontium on the absorption of rubidium (×——×) as percentage of the absorption from a 0·4 meq./l. solution.

an appreciable amount of rubidium was lost into demineralized water but this was less than half that lost into the highest concentration of rubidium chloride. 90 per cent. of the rubidium previously absorbed by the disks was lost into 12·8 meq./l. rubidium chloride. It is clear that by far the greater part of the ions absorbed in the first few minutes after disks were placed into salt solutions were not freely diffusible but were held in a readily exchangeable form.

Just as competitive power varied between ions, so did the extent of exchange when different ions were used in the type of experiment described above. When disks were transferred from radioactive strontium after 5 minutes into non-radioactive solutions of strontium, magnesium, calcium, or barium, most radioactivity was lost to the barium solution, least to the magnesium (Table II).

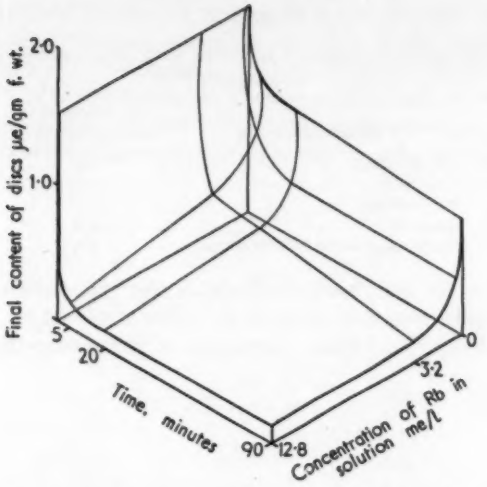


FIG. 2. The effect of time and of the concentration of rubidium on the release of rubidium from disks previously immersed in 0·2 meq./l. rubidium chloride for 5 minutes.

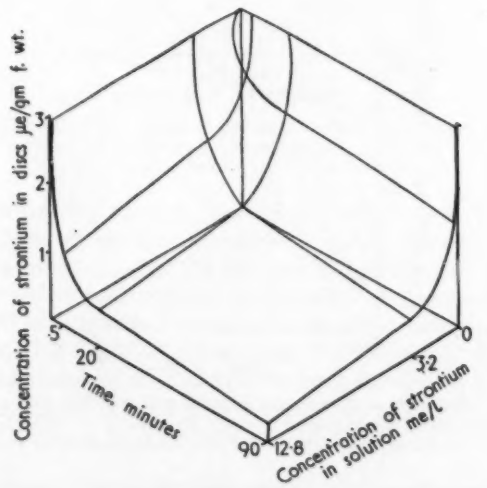


FIG. 3. The effect of time and of the concentration of strontium on the release of strontium from disks previously immersed in 0·4 meq./l. strontium chloride for 5 minutes.

TABLE II

The release of strontium previously absorbed into solutions of bivalent cations (0·4 meq./l.)

Exchanging ion	% released into solution
Magnesium	64·5
Calcium	58·9
Strontium	57·2
Barium	54·6
Least significant difference	0·57

Relationship of the two phases of uptake. A clear distinction can be drawn between the initial step and subsequent active transport by virtue of their different reactions to inhibitors. The nature of ionic competition also differs

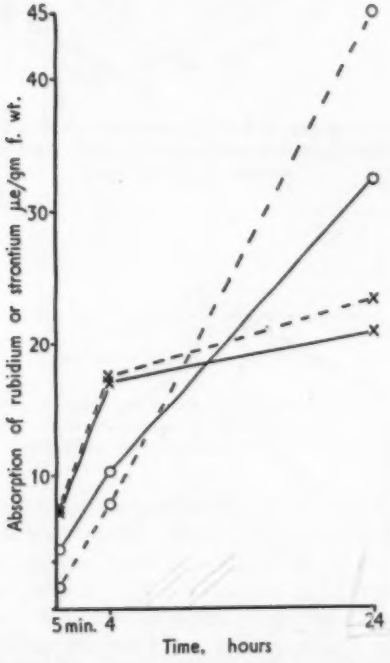


FIG. 4. The effect of time on the interaction of rubidium and strontium in absorption by carrot disks. (○——○ Rb alone, ○— —○ Rb with Sr, ×——× Sr alone, ×— —× Sr with Rb. All concentrations 3·2 meq./l.)

between the two phases. It is known that plants will absorb certain ions in preference to others (Collander, 1941) but it has been shown that the rate of absorption in the initial stages of uptake depends largely on atomic number and valency. The presence of strontium greatly reduces the uptake of rubi-

dium by carrot disks but over longer periods the presence of strontium or calcium is known to stimulate potassium uptake by barley roots (Viets, 1944).

The absorption of strontium and rubidium from strontium chloride, rubidium chloride, and an equi-equivalent mixture of the two, was measured after 5 minutes, 4 hours, and 24 hours. To prevent depletion of the solution in the relatively long periods of absorption used in this experiment the concentration

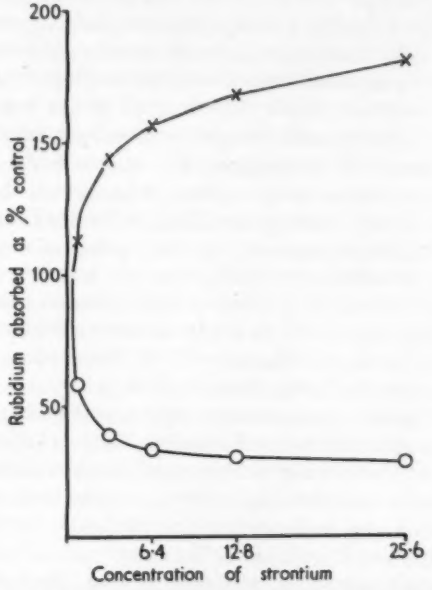


FIG. 5. The effect of a range of concentrations of strontium (meq./l.) on the absorption of rubidium by carrot disks in 5 minutes (○——○) or 24 hours (×——×).

of all salts was raised to 3·2 meq./l. It was found that the rate of absorption of strontium greatly exceeded that of rubidium after 5 minutes but declined rapidly with time (Fig. 4). After the first few hours, however, rubidium was absorbed more rapidly than strontium with only a slight diminution in rate up to 24 hours. The presence of rubidium increased the absorption of strontium slightly after 24 hours, but this effect was not significant. Strontium depressed the absorption of rubidium markedly in the early stages but caused a considerable acceleration subsequently. After 24 hours the absorption of rubidium was stimulated by strontium 40 per cent. above the level reached in rubidium alone.

When the concentration of strontium was varied up to 12·8 meq./l. with Rb at 3·2 meq./l. (Fig. 5) stimulation of up to 80 per cent. occurred after 24 hours, in spite of the reduction of over 60 per cent. which occurred in the first five minutes.

DISCUSSION

The nature of the mechanism responsible for the initial entry of ions into plant tissues has excited considerable discussion in recent years. Entry by diffusion into a free space in the tissue, by exchange with ions already present in the tissue, and as a result of equilibria of the Donnan type have all been postulated.

Hope and Stevens (1952) and Butler (1953) regarded the reversible uptake and loss of ions which occurs when roots are transferred between solutions of different concentrations as evidence of diffusion. Hope and Stevens (1952) also regarded the potentials which they observed on the root surface as diffusion potentials, though they were thought to result from a differential mobility of cations and anions in the protoplasm. The existence of a large preponderance of non-mobile anions in the protoplasm led Lundegårdh (1940, 1949) and Vervelde (1953) to regard these potentials as a result of Donnan equilibria. Such equilibria had been suggested by many previous observations. Petrie (1939) found that the effect of pH on ion uptake was that to be expected if there were an equilibrium of a Donnan type between tissue and solution. Stiles and Skelding (1940) similarly interpreted the ion movements in freshly cut disks of storage tissue and Elgabaly and Wiklander (1949) the absorption of sodium and calcium by barley roots from clays.

Much evidence is also available with regard to the occurrence of the exchange of cations between tissue and solution. Redfern (1922) found that calcium ions were absorbed in excess from calcium chloride by pea and maize roots and were replaced in the solution by other ions from the roots. Similar results were obtained by Stiles (1924) and Skelding and Rees (1952) with storage tissue disks, and Hoagland and Broyer (1942) with barley roots. It is possible to show that roots have hydrogen ions on their surface available for exchange (Williams and Coleman, 1950; Graham and Baker, 1951), and the total cation exchange capacity can be estimated by titration after electrodialysis. Russell and Ayland (1955) stressed the importance of exchange reactions in the initial step of entry. This has been further demonstrated for both uni- and bi-valent cations in the present investigation. It has been shown that the relative competing power of ions depends on atomic weight and valency. Of the univalent ions, caesium competes to the greatest extent and lithium the least. Similarly, among bivalent ions, barium is most effective in competing with strontium while magnesium shows the smallest effect. Bivalent ions are absorbed to a greater extent than univalent ions in the initial step and have greater competing power. This result is in agreement with those of Williams and Coleman (1950) and Lundegårdh (1940) who showed that the competing power of ions in absorption by barley or wheat roots respectively was in the order of the lyotropic series. Clear evidence of exchange reactions is also given by studies in which tissues were placed first into radioactive solutions and then into water or carrier solutions. Some cations were lost into distilled water but considerably more into salt solutions.

The apparent conflict between workers in this field who laid emphasis either on exchange or diffusion has lately been clarified by Hope and Robertson (1956). They have pointed out that diffusion and exchange are two aspects of one system, namely a Donnan equilibrium; diffusion predominates when the external concentration is high relative to the concentration of immobile ions, while exchange predominates when the external concentration is low. Accordingly it is not surprising that workers employing relatively high concentrations (10–20 meq./l.) have considered that entry shows the characteristics of diffusion (Butler, 1953; Sutcliffe, 1954; Epstein, 1956) while the importance of exchange has been emphasized by those using low concentrations (Russell and Ayland, 1955).

In assessing the relative importance in the life of the plant of entry by diffusion or exchange, consideration must be given to the conditions under which plants normally grow. The ionic concentrations which promote good growth of plants in water culture (Hoagland, 1940) or in the soil (Russell, 1950) are low relative to the estimates made of the concentration of indiffusible anions in the apparent 'free-space' of plant roots (Hope and Robertson, 1956; Vervelde, 1953). Under normal conditions the exchange component may be expected to be considerable and the extent of diffusion into a 'free-space' rather limited. Low concentrations cannot be used to estimate the 'free-space' of plant roots because of this considerable exchange of ions which takes place. Estimates made with the aid of higher concentrations may be of doubtful significance in view of the possibility that the permeability and other characteristics of cytoplasm may be greatly affected under such conditions.

The fate of the ions absorbed in the initial step may now be considered. A recent publication (Sutcliffe, 1957) has described ions absorbed in the initial step as being 'fixed' or 'bound'. Such terminology can be misleading, since ions taking part in the Donnan equilibrium remain freely mobile while maintaining a higher concentration internally owing to the excess of negative charges or immobile ions. This is shown in Figs. 2 and 3. When disks were transferred to a non-radioactive solution previously absorbed radioactive ions proved freely exchangeable. Recent discussions of the relationship of the initial step to subsequent active transport (Epstein, 1956; Sutcliffe, 1957) are also clarified by the concept of the continual passage of ions in both directions even though an overall unidirectional net movement may be taking place. The external solution, the cell walls, the intercellular spaces, and the cytoplasm must all be regarded as part of a complex system between the phases of which continual movement of ions occurs. These phases are in a state of dynamic equilibrium and no particular ion can be regarded as a component of any one phase to the exclusion of all others. Nevertheless it appears that a high concentration of ions in the initial step is not a prerequisite of rapid active transport since a reduction in the initial absorption of rubidium is accompanied by an increase in its rate of subsequent transport (Fig. 5).

Experiments in which the exchange of previously absorbed ions is measured encourage the classification of ions into three categories.

(*a*) 'Freely diffusible ions' which are presumed to be in the 'free spaces' and are readily lost into water.

(*b*) 'Exchangeable ions' which do not pass out into water but are displaced by the application of salts. These are presumably in the Donnan system.

(*c*) 'Bound ions' which are not displaced by either treatment and are assumed to have been actively transported (Epstein and Leggett, 1954).

This classification must, however, be employed with some caution. Thus in the experiment described in Fig. 2, 1·6 meq. of rubidium was absorbed from 0·4 meq./l. rubidium chloride. Assuming the 'free' space of 1 g. of carrot tissue to be equal to 0·25 ml., only 0·1 μ could have entered by diffusion; yet 0·8 μ are lost into water. Thus, this rubidium as well as that fraction lost in salt solutions must be presumed to have entered by exchange. It is possibly lost by exchange for hydrogen ions in the water (pH 5–6). The absence of any loss of strontium into water may be due to its greater exchange power compared with that of rubidium. Thus the extent of entry by diffusion in this experiment may be even smaller than would at first appear.

A small fraction of the absorbed ions are not displaced by exchange even after 90 minutes. In the case of rubidium, exchange curves became asymptotic in this time period; with strontium there is a suggestion of a slow continuing exchange. It is unlikely that in such tissue these apparently non-exchangeable ions are in superficial positions in the cytoplasm. They may be restrained in complexes from which they are released only slowly or they may be transported to some remote region of the cell, such as the vacuole. The more obvious non-exchangeability of the univalent ion may be due to its greater mobility and the readiness with which it enters processes of active transport.

Differences in behaviour of univalent and bivalent ions may depend in part on the extent to which they have penetrated the exchange system. It may be profitable to consider in this light the interaction of rubidium and strontium in periods of 5 minutes and 24 hours (Fig. 4). In 5 minutes markedly more strontium than rubidium entered the tissues. This effect is attributable to the greater exchange power of the bivalent strontium ion. Over the longer period the entry of rubidium exceeded that of strontium and its entry was increased in the presence of strontium. This stimulation is similar to that recorded by Viets (1944). Overstreet, Jacobsen, and Handley (1952) have suggested that bivalent ions stimulate the entry of univalent ions by participating in the mechanisms whereby rubidium is bound within cells. An alternative interpretation is suggested by the parallel which exists between these results and the type of interaction between uni- and bivalent cations which can occur in simple cation exchange-columns. By virtue of its greater ionic size the bivalent ion competes successfully for sites at the initial point of entry thus displacing univalent ions from such sites or preventing their retention, so that their movement down the column is accelerated. *The characteristic of ions which favours their retention in exchange columns at the site of entry is therefore antithetic to that which favours their onward movement.* Similarly in plant tissues the characteristic of ions which favour retention in roots (i.e. high atomic

weight or valency) are antithetic to those which favour their eventual accumulation (i.e. low atomic weight or valency). This concept appears helpful in the interpretation of the present data.

The consideration of a number of observations by previous workers is also simplified by applying this model. A number of multivalent ions, e.g. aluminium, cerium, and lanthanum, have been shown to stimulate the entry of univalent ions in the same manner as calcium (Fawzy, Overstreet, and Jacobsen, 1954). If an interpretation similar to that advanced by Overstreet, Jacobsen, and Handley (1952) is to be applied, the trivalent ions must be regarded as behaving similarly to calcium or strontium in mediating the entry of univalent ions; such a situation would seem surprising. On the present postulate, however, these effects are readily explained. The conclusion (Epstein and Hagen, 1952) that potassium competes with rubidium in a manner described as 'competitive inhibition' when the concentrations of both ions are similar, whereas sodium only behaves in this way at much higher concentrations may be explicable in similar terms. The concentration of sodium necessary to displace a given quantity of rubidium from synthetic exchange resins can be considerably greater than that of potassium necessary to produce the same effect (Kreesman, 1949); thus Epstein's observation may be evidence of competition in series of exchange systems.

While the interaction of cations both on entry into roots and on transfer across them is compatible with this concept, the overall process of active transport cannot be explained solely in these terms. The transport of ions in such a manner would not involve the expenditure of metabolic energy and the outstanding characteristic of active transport is the accumulation of ions against concentration gradients, mediated by energy released in respiration. Thus whereas the behaviour of ions in the initial process of entry (i.e. experiments of 5 minutes' duration here described) is compatible with a simple process of ion exchange some subsequent step which is energy dependent must occur during active accumulation (i.e. the experiments of 24 hours' duration). The manner in which the expenditure of energy could induce unidirectional transport against concentration gradients merits consideration. A flow of ions across an exchange system could be promoted by the creation of a suitable potential difference between its two sides. Similarly, a flow of ions could be induced along a diffusion path if the static sites are destroyed and recreated in a non-random manner. For example, if a series of negatively charged static sites 1 ... n is visualized, and each site is destroyed and recreated in sequence from 1 to n, positively charged ions will be moved from the sites of high number to those of low number provided that the acceptors are available for the ions released from site 1. A 'ladder' of this type could effect the accumulation of cations provided that:

(i) It traverses a medium which offers a high resistance to the free diffusion of cations, and

(ii) anions are conveyed either by an analogous mechanism or can diffuse passively by paths spatially separated from the 'ladder'.

In such a system the relative rates of movement of different ions would be the same as in the simpler case of an ion exchange system which has already been discussed. No binding reactions with specific carriers would be involved in a mechanism of this type.

While there is no direct evidence for its existence it is readily reconcilable with current concepts of the cytoplasm. There is considerable evidence that the tonoplast is a high resistance boundary to the movement of free ions and the growth of the cell to a predictable shape is indicative of a spatial organization of molecules and hence of charged sites, which, however, is as yet unidentified; furthermore there is abundant evidence that the turnover of charged sites on macromolecules is a general characteristic of metabolic processes. Some similarity exists between this hypothesis and that of Lundegårdh (1940) from which, however, it differs in a number of important respects. Emphasis is here laid on the possibility that active transport is a consequence of the *spatial orientation* of sites at which metabolic reactions occur on macromolecules in the cytoplasm. This viewpoint is offered as an alternative to current interpretations which involve the postulation of diffusable carrier substances and apparently envisage active transport to be largely independent of other cytoplasm functions.

ACKNOWLEDGEMENTS

This work was made possible by a grant-in-aid from the Agricultural Research Council to whom the authors express their thanks.

SUMMARY

The absorption of cations by carrot disks in periods of 5 minutes to 24 hours is described. It is shown that in short periods of absorption the competitive power of ions increased with atomic number and valency. Thus the absorption of rubidium is inhibited by the presence of strontium in short periods. However, in experiments lasting 24 hours the absorption of rubidium is greatly stimulated in the presence of strontium. Ions absorbed in short periods do not diffuse readily into distilled water but are largely exchangeable with other ions in the solution. These facts are discussed in relation to current theories of ion absorption.

LITERATURE CITED

Broyer, T. C. (1951). The nature of the process of inorganic solute accumulation in roots. In *Mineral Nutrition of Plants*, Ed. Truog. University of Wisconsin.

Butler, G. W. (1953). Ion uptake by young wheat plants. III. Phosphate absorption by excised roots. *Physiol. Plantarum.* **6**, 637.

Collander, R. (1941). Selective absorption of cations by higher plants. *Plant Physiol.* **16**, 691.

Elgabaly, M. M., and Wiklander, L. (1949). Donnan Equilibria in plant nutrition. I. Effect of acidoid content of plant on uptake of sodium and calcium from bentonite solutions. *Ann. Roy. Agr. Coll. Swed.* **16**, 334.

Epstein, E. (1956). Mineral nutrition of plants. Mechanisms of uptake and transport. *Ann. Rev. Plant Physiol.* **7**, 1.

—— (1956). Passive permeation and active transport of ions in plant roots. *Plant Physiol.* **30**, 529.

—— and Hagen, C. E. (1952). A kinetic study of the absorption of alkali cations by barley. Ibid. **27**, 457.

—— and Leggett, J. E. (1954). The absorption of alkaline earth cations by barley roots: Kinetics and Mechanisms. *Amer. J. Bot.* **41**, 785.

FAWZY, H., OVERSTREET, R., and JACOBSEN, L. (1954). The influence of hydrogen ion concentration on cation absorption by barley roots. *Plant Physiol.* **29**, 234.

GRAHAM, E. R., and BAKER, W. L. (1951). Ionic saturation of plant roots with special reference to hydrogen. *Soil Science*, **72**, 435.

HOAGLAND, D. R. (1940). *Inorganic plant nutrition.* Chronica Botanica Co. U.S.A.

—— and BROYER, T. C. (1942). Accumulation of salt and permeability in plant cells. *J. Gen. Physiol.* **25**, 865.

HOPE, A. B., and STEVENS, P. G. (1952). Electrical potential differences in bean roots and their relation to salt uptake. *Aust. J. Sci. Res.* **5**, 335.

—— and ROBERTSON, R. N. (1956). Initial absorption of ions by plant tissue. *Nature*, **177**, 43.

KREESMAN, T. R. E., and KITCHENER, J. A. (1949). Cation exchange with a synthetic polysulphonate resin. Part I. Equilibria with univalent cations. *Journ. Chem. Soc.* 1790.

LUNDEGÅRDH, H. (1940). On the absorption and accumulation of inorganic ions. *Ann. Agric. Coll. Sweden*, **8**, 234.

—— (1949). The time course of ion absorption of wheat roots and the influence of the concentration. *Physiol. Plantarum.* **2**, 388.

MARTIN, R. P., and RUSSELL, R. S. (1950). Studies with radioactive tracers in plant nutrition. II. The estimation of radioactive tracers. *J. Exp. Bot.*, **1**, 141.

OVERSTREET, R., JACOBSEN, N. L., and HANDLEY, R. (1952). The effect of calcium on the absorption of potassium by barley roots. *Plant Physiol.* **27**, 583.

PETRIE, A. H. K. (1939). The intake of ions by carrot tissue at different hydrogen ion concentrations. *New Phytol.* **37**, 211.

REDFERN, G. M. (1922). On the absorption of ions by roots of living plants. 1. The absorption of the ions of calcium chloride by pea and maize. *Ann. Bot.* **36**, 167.

ROBERTSON, R. N., WILKINS, M. J., and WEEKS, D. C. (1951). Studies in the Metabolism of Plant Cells. IX. The effects of dinitrophenol on salt accumulation and salt respiration. *Aust. J. Sci. Res.* **4**, 248.

RUSSELL, E. J. (1950). *Soil conditions and plant growth.* Eighth edition. Longmans Green & Co. London.

RUSSELL, R. S. (1954). The relationship between metabolism and the accumulation of ions by plants. *Symposia of the Society for Experimental Biology*, **8**, 343.

—— and AYLAND, M. J. (1955). Exchange reactions in the entry of cations into plant tissues. *Nature*, **175**, 204.

SKELDING, A. D., and REES, W. J. (1952). An inhibitor of salt absorption in the root tissues of red beet. *Ann. Bot.* N.S. **16**, 573.

STEWARD, F. C., and HARRISON, J. A. (1939). The absorption and accumulation of salts by living plant cells. IX. The absorption of rubidium bromide by potato discs. Ibid. **3**, 428.

—— and MILLAR, F. K. (1954). Salt accumulation in plants. A reconsideration of the role of growth and metabolism. *Symposia of the Society for Experimental Biology*, **8**, 367.

STILES, W. (1924). The absorption of salts by storage tissues. *Ann. Bot.* **38**, 617.

—— and DENT, K. W. (1946). Further observations on the absorption of manganese chloride by storage tissue. Ibid. **10**, 203.

—— and SKELDING, A. D. (1940). I. The salt relations of plant tissues. The absorption of potassium salts by storage tissue. Ibid. **4**, 329.

SUTCLIFFE, J. F. (1954). The exchangeability of potassium and bromide ions in cells of red beetroot tissue. *J. Exp. Bot.* **5**, 313.

—— (1957). The selective uptake of alkali cations by red beet tissue. Ibid. **8**, 36.

WILLIAMS, D. E., and COLEMAN, N. T. (1950). Cation exchange properties of plant surfaces. *Plant and Soil*, **2**, 243.

USSING, H. H. (1949). Transport of ions across membranes. *Physiol. Rev.* **29**, 127.

VERVELDE, G. T. (1953). The Donnan principle in the ionic relations of plant roots. *Plant and Soil*, **4**, 309.

VIETS, F. G. (1944). Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. *Plant Physiol.* **19**, 466.

Exchangeable Ions in Beet Disks at Low Temperature

G. E. BRIGGS, A. B. HOPE,[1] AND M. G. PITMAN

Botany School, Cambridge

Received 22 August 1957

SUMMARY

The relatively rapid passage of ions into the 'Free Space' of disks of beetroot has been studied using radioactive tracers while active accumulation was reduced to a negligible proportion by using 'salt saturated' tissue at 2° C.

The experiments confirm the suggestion made previously that the free space can be treated as if made up of two main components, the 'Water Free Space' (W.F.S.), where the concentrations of anion and cation quickly become equal to those of the external solution, and the 'Donnan Free Space' (D.F.S.), containing a high concentration of non-diffusible anions.

In disks pretreated with solutions of RbI to remove all other mobile ions from the free space the amount of exchangeable I and Rb was measured by the uptake of I^{131} and Rb^{86} at various external concentrations. The excess of cations over anions (the 'extra exchangeable Rb') was used as an estimate of the amount of non-diffusible anions in the D.F.S. This was approximately constant at 10–13 m.equiv./kg. A gradual rise in the extra exchangeable Rb as the external concentration rose from 1 to 20 m.equiv./l. has been explained as consistent with the Donnan anions having arisen from weak acids with a pK of about 3.

The volume of the D.F.S. was estimated from the amount of extra exchangeable Rb in disks which had previously been treated so that the counterions in the D.F.S. were exclusively Ca, and which were subsequently brought to equilibrium with various concentrations of RbBr. The mean volume from four experiments was 2·1 per cent. so that the concentration of non-diffusible anions in the D.F.S. was 560 m.equiv./l. In consequence the fraction of the exchangeable anion in the Donnan part of the free space is negligible and so the amount in the free space divided by the external concentration gives an estimate of the volume of W.F.S. as 200 ml./kg.

The results are compared with earlier estimates of the non-diffusible anions concentration made by different means. In considering the location of the D.F.S. in the tissue, account must be taken of the fact that the area of the cell protoplasts considered as smooth spheres is much too small to contain the number of immobile anions present (*c.* 12 m.equiv./kg) since there would be less than 1 $Å^2$ for each ion. For reasons given, the D.F.S. is thought to be mainly in the cell cytoplasm, a layer 1 micron thick in cells of diameter 120 microns contributing the required volume.

INTRODUCTION

It has been suggested that the uptake of salts by slices of plant tissue can be analysed into a relatively rapid passage into what has been called the free

[1] Present address: Plant Physiology Unit, Division of Food Preservation and Transportt C.S.I.R.O., and Botany School, University of Sydney.

space and a slower accumulation into the vacuole (see reviews by Robertson, 1951; Epstein, 1956). The latter process can be reduced to relatively small dimensions by lowering the temperature to near 0° C. and by using 'salt saturated' tissue (Sutcliffe, 1952); treatments which have little effect on the passage into the free space. This state of affairs not only suggests that this passage is dependent mainly upon physical factors such as diffusion, but also enables easier examination of this process.

It has been shown elsewhere (Briggs and Robertson, 1957) that the uptake into the free space can be explained if it is assumed that one part of the free space behaves as water, in that it has the same concentration of anions and cations as the external solution, and that the other part behaves as a Donnan system containing non-diffusible anions. If the concentration of the latter is high relative to the external concentration of salt then the diffusible anions in the Donnan system (Donnan Free Space, D.F.S.) can be neglected and hence the amount of anions in the free space as a whole divided by the external concentration provides an estimate of the volume of the other part of the free space (Water Free Space, W.F.S.): Epstein (1955) uses the term 'outer space'. The excess of cations over anions can then be used as an estimate of the amount of non-diffusible anions in the Donnan system.

The present paper presents and interprets on the above basis the results of experiments with disks of beetroot in solutions containing rubidium labelled with Rb^{86}, calcium labelled with Ca^{45}, bromide labelled with Br^{82}, and iodide labelled with I^{131}.

EXPERIMENTAL METHODS

Material. Disks from the root of red beet (*Beta vulgaris*), 1 mm. thick and 15 mm. in diameter were cut with a microtome, rinsed twice in distilled water, and placed with distilled water in a Florence flask to be aerated and gently agitated by a moist, clean, air stream. After 4 days a solution of RbBr or RbCl, of about 10 m.equiv./l. was substituted for the distilled water and renewed twice during the next 4 days. The weight of the disks was 20–40 g. and the solution volume 500 ml. The temperature varied between 17° and 20° C.

Preparation of the isotopes. Rb^{86} was received from Harwell as the carbonate. It was dissolved in about 30 ml. of water, converted to the bromide or the chloride by the addition of excess of the appropriate acid and then evaporated to dryness, redissolved, and after further evaporation was made up to a solution containing 5–7 m.equiv./l. Rb with an activity of about 6 μC./ml.

Br^{82} was received as the ammonium salt and was diluted with carrier RbBr solution to give solutions of suitable counting rates. At higher concentrations the rubidium concentration was far in excess of the ammonium, but at the lower concentrations (0·5–1·0 m.equiv./l.) the ammonium was appreciable. The uptake of the bromide in these experiments was found not to be dependent on the nature of the accompanying cation, whether K, NH_4, or Rb.

High specific activity Ca^{45} was obtained as the chloride and the required dilutions made with inactive $CaCl_2$.

'Carrier Free' I^{131} in dilute Na_2SO_3 was diluted with KI to make the labelled solutions. The amount of Na_2SO_3 in the final solutions was negligible.

Estimation of Rb, Br, and I. The amount of Rb, Br, and I, in 5 disks was estimated by directly counting each beet disk placed with repeatable geometry on a perspex tray under an aluminium end-window Geiger tube. To prevent drying and warming to room temperature the disks were counted for only 40 sec.; counting rates (Y) from 3,000 to 5,000 counts/min. were aimed at. The counting rates were related to the absolute amount of ion present by counting a 'disk' of radio-active solution of known concentration with the same dimensions as the beet disk. The liquid disks were counted under the same conditions as the beet disks, the solution being contained in a perspex ring 1 mm. in thickness on the perspex tray mentioned above. The meniscus was kept level by putting a strip of filter paper 1 mm. wide round the edge of the perspex ring. Absorption of β-rays in the beet and water would be negligible in the case of Rb^{86} (80 per cent. 1·8 MEV max. energy), and, while appreciable in the case of Br^{82} (0·465 MEV max. energy) and I^{131} (mainly 0·61 MEV), energy absorption would be similar for beet and water. The sensitivity of the method was between 10^{-12} and 10^{-9} Equiv. per count/minute, depending on the concentration of salt in the medium.

Estimation of Ca. The amount of Ca in a number of disks (usually 10) after equilibration in a solution of $CaCl_2$ labelled with Ca^{45} was estimated from measurements of the activity of the medium before and after adding the tissue. Direct counting of the beet disks was not advisable as the β-rays from Ca^{45} are comparatively soft (0·25 MEV). Samples of the medium, 0·1 ml. in volume, were dried together with a few drops of dilute detergent on aluminium planchets. The effect of the detergent was to spread the calcium salt evenly and so minimize self-absorption of β-rays. The spreading was controlled by a ring of silicone varnish painted around the edge of the planchets. Five such samples were averaged for each determination. At the higher concentrations of $CaCl_2$ dilute ammonium oxalate solution was added to the planchets before drying, to precipitate the calcium in a non-hygroscopic form. If this was not done the $CaCl_2$ formed small droplets causing variable self absorption.

Experimental procedure. At the end of four days in the RbBr solution the disks had become almost 'salt-saturated' (see Sutcliffe, 1952). All disks used in a given experiment had the same accumulatory history up to the time of placing in fresh solutions of RbBr at a temperature of $2\pm1°$ C. and concentrations C_o varying from 0·5 to 50 m.equiv./l. The disks were first put in test-tubes and aerated at 2° C. in solutions of inactive isotope of concentration C_o. This treatment involved at least three successive changes of the solution and was designed to bring the free space of the tissue into equilibrium with the inactive solution. By continuously bubbling a gentle, moist, air stream through the solutions they were both aerated and stirred. When this treatment was finished the disks were blotted lightly to remove surface solution only and put in an active solution of the same total concentration as that from which they were taken. Blotting, which was done only at the beginning of an active

exchange or before counting a disk, was very light so as to remove the minimum of solution from the free space in the disk and an effort was made to standardize the method. The disks were left in the solution for a time sufficient for a steady state relation with the free space to be attained, but not long enough for appreciable exchange to occur with the vacuole (see results below). This time was determined by the behaviour of a suitable control or else, as in later experiments where greater accuracy was desired, determinations of the activity of the disks were made at intervals over a suitable period and the extrapolate to zero time was taken as the activity of the ion in the free space. Finally the disks were blotted and weighed.

Experimental errors. (a) *Disk counting.* The Standard Errors appearing in the graphs are the Standard Errors of the mean counting rate of five disks, calculated from $\sqrt{\left\{\sum \frac{(Y-\bar{Y})^2}{20}\right\}}$ and combined with the counting error in the calibration by taking the square root of the sum of the squares of the percentage errors. The random counting errors are included in the Standard Error of the mean counting rate but, since disks are counted for at least 3,000–5,000 counts, giving a random error of less than ± 2 per cent., they are much less than the variation in Y from other causes. With five such counts the Standard Error in the counting would be about $\pm 0{\cdot}4$ per cent. The other causes of variation in Y, the disk counting rate, were probably variations in xylem content and in blotting procedure. Young beetroots were chosen to reduce the former as much as possible.

(*b*) *Errors in Ca determinations.* In the estimations of exchangeable Ca, based on counting rate of the solution before and after adding tissue, the Standard Errors include both counting and pipetting variations, since the counting rates were the mean of five dried samples of 0·1 ml. of solution as described in Experimental Methods above. The Standard Error of the mean was here ± 1 per cent. but the Standard Error of the result is higher than this since the estimate of the amount in the disks involved differences between counting rates.

EXPERIMENTAL RESULTS

The exchangeable rubidium. At 2° C. the equilibration of Rb^{86} in the free space with that in the external medium when the disks are in equilibrium with inactive RbBr, 1 m.equiv./l., proceeds as shown in Fig. 1. This records the mean counting rate Y of five disks at different times after the addition of active isotope to the medium. The results suggest that the exchange has at least two phases, one nearly complete in about 90 minutes (Fig. 1*a*), and a very much slower exchange as shown in Fig. 1*b* where counts were made on a second set of disks over a longer time. In Fig. 1*b* the difference between the mean counting rate at 90 minutes and the extrapolate to zero time of the straight line portion of the graph is only 1·4 per cent. while the variation between disks is ± 3 per cent. Similar experiments were done using Br and I, where equilibration is faster, and using Ca, where equilibration is slower than with Rb.

Hence, in what follows, the 'amount of exchangeable ion' is that amount determined from the change in specific activity in either disks or medium at the end of a period of 90–100 minutes for Rb, 20–30 minutes for Br and I, and about 200 minutes for Ca.

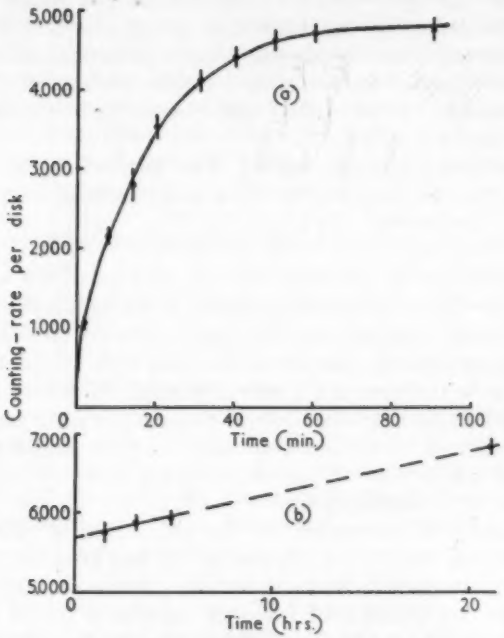


FIG. 1. Isotopic equilibration of disks in 1 m.equiv./l. RbBr solution labelled with Rb^{86}.

When the data from Fig. 1*a* are plotted in the logarithmic form

$$\log_{10}(1-Y/Y_\infty) \text{ v. } t,$$

where Y_∞, the value for Y at 'infinite' time, is taken as 4,900, the slow uptake being neglected, the result is Fig. 2. For a homogeneous disk, large in area relative to its thickness, $\log_{10}(1-Y/Y_\infty)$ should fall with time rapidly at first, reaching a value $-0{\cdot}3$ when $t = l^2/20D$, and then the slope should remain constant at $8D/2{\cdot}3l^2$, where D is the coefficient of diffusion and l is the thickness. For the curve in Fig. 2 the first equation gives a value for D of $1{\cdot}0\times 10^{-6}$ cm.^{2} sec.$^{-1}$, and the second $1{\cdot}2\times 10^{-6}$. It has been shown elsewhere (Briggs, 1957) why the geometry of the free space and other factors in the disk should result in an overall value of D less than it is in water at 2° C. (1×10^{-5}).

Fig. 3*a* shows the amount of exchangeable Rb, in m.equiv./kg. (fresh weight) plotted against the concentration C_0 of RbBr in the solution with

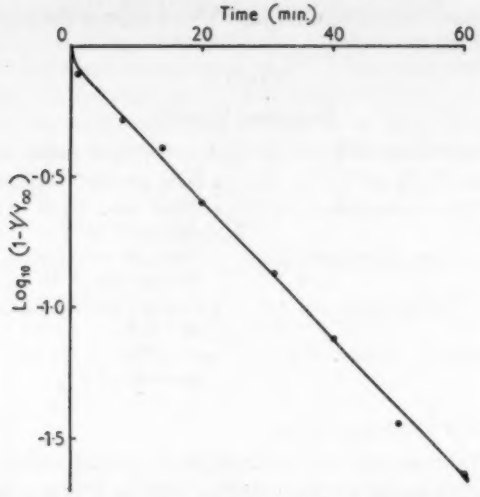


FIG. 2. The data from Fig. 1*a* plotted in the logarithmic form, $\log_{10}(1-Y/Y_\infty)$ v. t. Y = counting rate of disk at time t, Y_∞, counting rate at 'infinite' time.

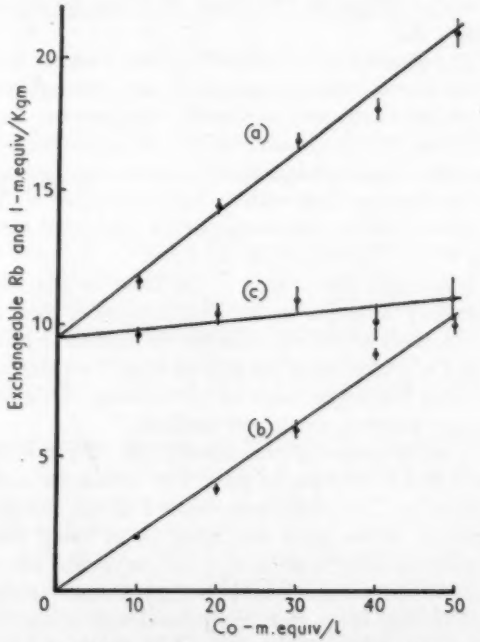


FIG. 3. (*a*) The amount of exchangeable Rb plotted against C_o. (*b*) The amount of exchangeable I plotted against C_o. (*c*) The difference between (*a*) and (*b*), or 'extra exchangeable Rb'.

which the free space was in equilibrium. The details of the pre-treatment are given in Table I (*a*).

TABLE I

Treatment of disks

(*a*) For exchangeable Rb	(*b*) For exchangeable Ca
1. 4 Days aer. dist. H_2O, 20° C.	4 Days aer. dist. H_2O, 20° C.
2. 4 Days aer. RbBr, 10 m.equiv./l., 2 renewals, 20° C.	4 Days aer. RbBr, 10 m.equiv./l., 2 renewals, 20° C.
3. 3 Changes aer. RbBr, 50 m.equiv./l., 30 min. ea., 2° C.	3 Changes aer. $CaCl_2$, 20 m.equiv./l., 30 min. ea., 2° C.
4. 3 Changes aer. RbBr, concn C_o, 30 min. ea., 2° C.	3 Changes aer. $CaCl_2$, concn C_o, 30 min. ea., 2° C.
5. aer. $Rb^{86}Br$, concn C_o, 90 min., 2° C.	aer. $Ca^{45}Cl_2$ or $Rb^{86}Br$, concn C_o, 150–200 min. 2° C.

The exchangeable bromide and iodide

Fig. 3*b* shows the amount of exchangeable I plotted against C_o measured on five-disk samples similar to those used for Fig. 3*a*. It is seen that the amount of exchangeable I is closely proportional to external concentration. This is what would be expected if the I was present as RbI in a 'water phase' of the free space, the volume of this W.F.S. being about 200 ml./kg. Similar results were obtained using Br.

Curve (*c*) in Fig. 3 shows the Rb in the free space in excess of that associated with I. This excess shows little change with C_o and is equal to 10·5 m.equiv./kg. In other experiments the excess, which it is proposed to call the 'extra exchangeable Rb', was 11–13 m.equiv./kg. This is consistent with this fraction of the Rb being associated with indiffusible anions in a Donnan system, the equivalent amount changing little with C_o as long as $C_o \ll A$. The concentration, A, of the non-diffusible anions cannot be calculated from the amount until the volume of the Donnan phase is known.

It is possible to estimate the volume of the Donnan phase by introducing a further variable into the experiment. This consists of measuring the extra exchangeable Rb in disks which have had the exchangeable cations in the free space replaced by Ca before being put into labelled Rb solution. This experiment also illustrates the importance of considering the effect of bivalent cations in exchanges between tissue and medium.

The effect of Ca on the amount of exchangeable Rb. The amount of exchangeable Rb was measured in two sets of disks from the same beetroot but with different pretreatments. The disks were treated as set out in Table I. The procedure in part (*a*) of the table was designed to bring the free space to equilibrium with Rb of concentration C_o (inactive) before the disks were put into labelled solution of total concentration C_o, as in the experiment recorded in Fig. 3. In part (*b*) the disks were first treated with a solution of $CaCl_2$ in such a way as to replace all exchangeable cations in the free space by Ca and then the free space was brought into equilibrium with a concentration of

$CaCl_2$ of concentration C_o. The disks were then put, as before, in labelled Rb, concentration C_o. On other disks from the same tissue an estimate of the water free space was made with the aid of I^{131}. Using this estimate the 'extra exchangeable Rb', in disks (*a*) and (*b*) was calculated.

The results of one such experiment are shown in Fig. 4 *a* and *b*. The disks pretreated with Ca (curve (*b*)) have less extra exchangeable Rb than those pre-

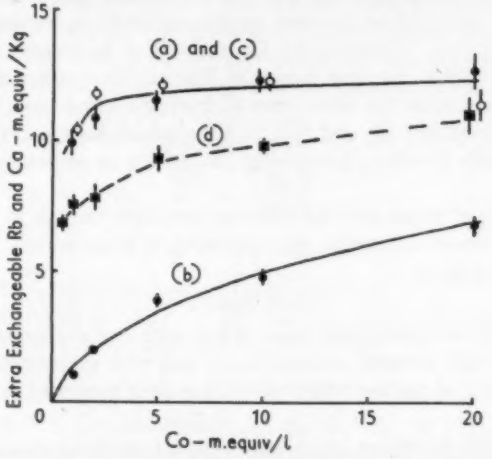


FIG. 4. (*a*) Closed circles. Extra exchangeable Rb in tissue pretreated with Rb.
(*c*) Open circles. Extra exchangeable Ca in tissue pretreated with Ca.
(*b*) Extra exchangeable Rb in tissue pretreated with Ca. The line indicates theoretical values (cf. p. 137).
(*d*) Extra exchangeable Rb in tissue not pretreated to ensure complete removal of the bivalent cations.

treated with Rb (curve (*a*)), but the difference becomes smaller as C_o increases. The rise in curve (*a*) from 10 to 12·7 as C_o rises from 1 to 20 m.equiv./l. is discussed later. A quantitative analysis of the effect of Ca on the exchangeable Rb is given later and reveals that just such amounts of Rb exchanging for Ca would be expected in a Donnan system with A equal to about 600 m.equiv./l. The relative volume occupied by this phase is thus about 12/600 or 2 per cent. of the tissue.

The effect of the pretreatment in determining the amount of a known species of ion in the Donnan phase is illustrated in the dotted curve, (*d*), in Fig. 4, which shows an earlier experiment in which the pretreatment with RbCl solutions was not sufficient to remove all bivalent cations from the Donnan phase. The pretreatment of Table I (*a*) was followed except that step 3 was omitted. It is seen that the extra exchangeable Rb is not constant, but rises from about 7 m.equiv./kg. at $C_o = 1$ to 11 at $C_o = 20$ m.equiv./l. Compared with curve (*a*) in Fig. 4, for which step 3 was included, the amount of extra exchangeable Rb is less the lower the external concentration.

If curve (*b*) in Fig. 4 does in fact represent increasing amounts of Rb exchanging with Ca already in the Donnan phase, it is of interest, and a further test of the hypothesis, to examine whether the extra exchangeable Ca in disks pretreated with Ca solutions is equal to the extra exchangeable Rb in disks pretreated with Rb.

The amount of exchangeable Ca in disks pretreated with Ca. Disks with the same pretreatment as disks (*b*) in Table I, but placed finally in $CaCl_2$ instead of RbBr, were used to find the total exchangeable Ca at external concentrations 1–20 m.equiv./l. Corrected for Ca in the W.F.S., the results of such an experiment are plotted as open circles in Fig. 4*a*. They are comparable with curves (*a*) and (*b*) since the disks were all from the same root. It is seen that the extra exchangeable Ca and Rb in disks which have received sufficient pretreatment with solutions containing Ca and Rb respectively is practically the same.[1]

The accuracy of these experiments was not high enough to detect differences less than about 1 m.equiv./kg. and hence it is not certain that there are no specific differences.

DISCUSSION

In the experiments described above the amounts of exchangeable ions and their variation with external concentration and with pretreatment lend support to the model of the beet disks which has been proposed and which is as follows:

(*a*) Ions are able to diffuse quickly into the intercellular spaces, which have become injected with water during the time in aerated distilled water and salt solution, and into water spaces in the cell walls. This space has been termed the 'water free space' (W.F.S.) since the rate of equilibration when a radioactive isotope of Br or I exchanges with the stable isotope suggests a diffusion coefficient comparable with that for Br or I in water. The apparent size of W.F.S. in 1 mm. thick beetroot disks is 15–20 per cent. by volume. When the external concentration is small relative to that of the non-diffusible anions in the Donnan Free Space (D.F.S.) then the diffusible anions in the latter are negligible and the apparent volume of the W.F.S. is equal to the Apparent Free Space (based on anion uptake) of Briggs and Robertson (1957), or the 'outer space' of Epstein (1955).

(*b*) Cations in the W.F.S. are able to exchange with cations already in a second phase which acts as a Donnan system (the D.F.S.) As shown in the analysis below the Donnan phase may be confined to 2–3 per cent. of the tissue volume. If care is taken to have only monovalent cations in this phase, as ensured by the treatment in Table I (*a*), the number of exchange sites is given by the amount of 'extra exchangeable Rb' (total exchangeable Rb minus exchangeable Br or I). This number is about 12 m.equiv./kg. and does not show much change with increasing C_o. The rise in extra exchangeable Rb from about 10 for $C_o = 1$, to 13 for $C_o = 20$ m.equiv./l. in Fig. 4*a* might be

[1] Since the isotopic equilibration with Ca is slower than with Rb extra time must be allowed for the former or the measured extra exchangeable cation will be too small.

due to increase in A from increase in ionization of an acid HA. If the outside pH is constant at 6 and the concentration of A is 500 m.equiv./l. when $C_o = 1$ m.equiv./l. then the pH in the D.F.S. will rise from 3·3 to 4·5 when C_o is increased from 1 to 20 m.equiv./l. and A will increase by 30 per cent. For such an increase the pK of the acid HA would be 2·8, whence it is almost completely ionized at pH 4·5.

On the other hand, when there are bivalent ions in the Donnan phase, consequent upon the previous treatment with solutions containing Ca or inadequate washing with solutions containing Rb, the amount of extra exchangeable Rb does not give a measure of the number of Donnan (indiffusible) anions (compare Fig. 4*b*).

In this case, there is an equilibrium between the Ca, Rb, and indiffusible anions such that

$$\frac{Ca_i}{Ca_o} = \left(\frac{Rb_i}{Rb_o}\right)^2 \tag{1}$$

and

$$A = Ca_i + Rb_i. \tag{2}$$

The symbols represent equivalent concentrations.

Equation (2) holds so long as the mobile anion concentration in the Donnan phase is negligible compared with A. Also

$$V_w C_o + V_D A = (V_o + V_w) Ca_o + V_D Ca_i, \tag{3}$$

where V_D, V_w, and V_o are the volumes of the D.F.S., W.F.S., and external solutions, respectively. C_o is the total concentration outside. In equation (3) the amount of Ca in the disks before immersion in RbBr is equated to that in the disks plus that in the medium after the new equilibrium is reached. Similarly the equation for conservation of Rb ion is

$$V_o C_o = V_D Rb_i + (V_w + V_o) Rb_o. \tag{4}$$

Putting $V_D A = a$, the amount of Donnan anion, and $V_D Rb_i = X$, the amount of Rb entering the D.F.S. in exchange for Ca, both of which are measured, and eliminating Rb_o, Ca_o, and Ca_i, the volume of the D.F.S. is

$$V_D = (V_o + V_W) \frac{X^2}{(V_o C_o - X)^2} \frac{V_W C_o + X}{a - X}. \tag{5}$$

From the mean results of four experiments, similar to the one giving the results plotted in Fig. 4*b*, where C_o was 1, 2, 5, 10, and 20 m.equiv./l. and a was 12 m.equiv./kg., the corresponding values of V_D are 21·6, 21·4, 23·2, 19·2, and 21·8 ml./kg.—the mean being 21·4±0·7. Thus A has a mean value of 12/0·0214 = 560 m.equiv./l. of D.F.S. Using this figure the predicted values for X as a function of C_o are as shown by the full line in Fig. 4*b*. It is seen that the values observed in the experiment recorded do not deviate markedly from the values so predicted.

This estimate of the volume of the D.F.S. refers to the volume of the solution and not to the whole phase including non-diffusible anions which may be large. It may be an over-estimate because the activity coefficients of the ions

are throughout assumed to be unity. Those affecting the X^2 term in the numerator and the $(a-X)$ in the denominator in equation (5) may be appreciably less than unity; the latter term, which refers to a bivalent cation, perhaps more so.

The effect of such a high concentration of immobile ions would be to keep mobile anions such as Br and I at a very low level in the Donnan phase. With an external concentration of uni-univalent electrolyte as high as 50 m.equiv./l. the concentration of diffusible anions in the D.F.S. will be only 4·15 m.equiv./l. and that of the cation 604, if A is 600. For a bi-univalent electrolyte the values are 14·3 and 614 m.equiv./l. Thus, for A as high as postulated, the total amount of exchangeable anion would not appear very different from that in the W.F.S.; the actual difference being too small to detect in experiments such as that recorded in Fig. 3. For example, if $C_o = 50$ m.equiv./l. the W.F.S. 20 per cent. and the Donnan volume 2·2 per cent., then the total exchangeable I would have been 10·0+0·09 = 10·09 m.equiv./kg. If, however, A were as low as 100, then the total exchangeable I would have been 10·0+2·67 = 12·67 m.equiv./kg., compared with the experimental finding of 10·0±0·33, and the difference should have been detectable. Hence, the results of measurements of the exchangeable I (and Br) are consistent with the hypothesis that $A \gg 100$ m.equiv./l. and the exchangeable anion is therefore a good measure of the anion (and of the cation) in the W.F.S.

With such a high concentration of non-diffusible anions balanced by diffusible bivalent cations the replacement of the bulk of the latter by equilibration with a solution containing univalent cations would require a very large volume or many repeated changes unless the concentration was very high. This would explain the apparent increase in number of exchange sites when C_o is increased from 1 to 20 m.equiv./l. in Fig. 4*d* compared with the approximately constant number in Fig. 4*a*. In the former experiment the disks had been in three changes of RbBr (about 10 m.equiv./l.) at 20° C. followed by three changes in concentrations C_o at 2° C. whereas the disks of Fig. 4*a* had received in addition three changes in 50 m.equiv./l. at 2° C. before being placed in concentration C_o (compare also Table I).

Fig. 5 shows the amount of a bivalent cation remaining in a Donnan system after successive equilibrations with a solution of a uni-univalent electrolyte deduced from equations (1) to (4) with $A = 600$ m.equiv./l., and $V_o/V_D = 600$. The original counterions are assumed to be exclusively bivalent. The graph shows that only 24 per cent. of the bivalent cation has been removed after five equilibrations with the concentration 1 m.equiv./l., giving a subsequently low value for the amount of exchangeable univalent cation such as Rb. When the concentration is 50 m.equiv./l. nearly all the bivalent cation has been removed by three equilibrations.

Most of the published data on the amounts of cations exchanged or taken up in the 'physical uptake' are unsuitable for estimating the number and concentration of exchange sites, because there is not sufficient information to provide a basis for calculating the univalent and bivalent cations in the D.F.S.

Briggs and Robertson (1957) have calculated A from the results of Stiles and Skelding (1940) with carrot disks in solutions of $MnSO_4$ where the counterions in the free space of the tissue may be assumed to have been all bivalent. Assuming the Donnan phase occupied as much as 5 per cent. of the tissue the concentration of A was 400–600 m.equiv./l.

The value for A of about 600 m.equiv./l. is very much higher than that suggested by some earlier investigators. For example, Tendeloo *et al.* (1946),

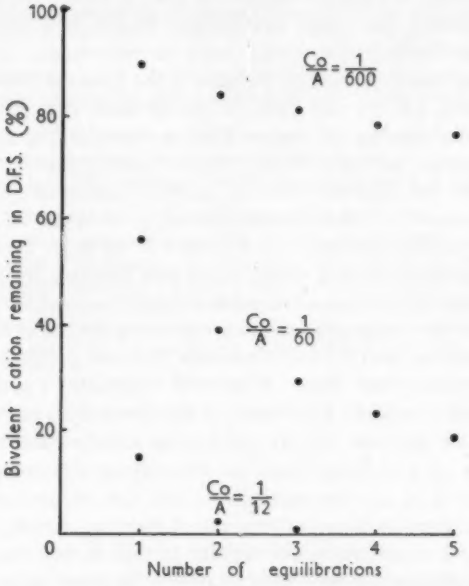


FIG. 5. The removal of bivalent cations from a Donnan system by successive equilibrations with a solution of uni-univalent electrolyte 600 times the volume of the Donnan system. A = concentration of non-diffusible anions, C_o = concentration of univalent cation.

in considering the variation of electric potential difference in roots with external concentration, calculated A to be about 40–90 m.equiv./l., and Hope (1953) estimated A to be about 10 m.equiv./l. from the increase in 'Chloride Free Space' with increase of C_o. However, as pointed out by Briggs and Robertson (1957), in making these estimates both authors failed to consider the effect of the presence of bivalent ions in the Donnan phase. Hope neglected the exchange of these bivalent cations with the K ions in the external solution. Hence some of the increase in 'Apparent Free Space' with increasing C_o was probably a reflection of the change in the conductivity due to an increase in the exchange of bivalent cations for K ion. The estimate of the diffusion constant from change of conductivity with time is also in error if cation exchange is not negligible. The experiments made by one of us (Hope, 1953)

with bean roots are therefore being repeated. The consequences of the neglect of bivalent cations left in the free space on estimates of the concentration of *A* from uptake and from potential differences are referred to in more detail by Briggs and Robertson (1957).

Finally, the location of the Donnan phase in the beet disks should be considered. There are two obvious possibilities, or a combination of the two, namely that the indiffusible anions are fixed either in surfaces or in a phase of much greater depth. We say greater, since even if these ions are in a surface they would influence the ionic distribution in the adjacent medium to a depth of perhaps 10 Å.

Using this figure and the relative volume of the Donnan phase suggested by the analysis above, i.e. 2·1 per cent., it can be seen that the area required would be $2{\cdot}1 \times 10^5$ cm.²/g. of tissue. This is considerably greater than the area of the protoplast surfaces considered as smooth spheres which would be 400 cm.²/g. if the cell diameter was 120 μ and 20 per cent. of the tissue was intercellular space. If all the Donnan ions (13 m.equiv./kg.) were on this surface, there would be two such ions in each Å² of area. This seems impossible. If a considerably greater surface area was invoked, for example, in the microfibrils of the cell walls, such a picture might be tenable. However, since it is known that the cytoplasm contains proteins, &c., which may ionize to give immobile anions, and for other reasons, it seems preferable to locate the bulk of the Donnan phase there. A layer of cytoplasm 1 μ thick in cells of radius 60 μ would constitute 4 per cent. of the tissue if 20 per cent. was intercellular space—2·1 per cent. (cf. p. 137) for the solution and 1·9 per cent. for solids. There is an additional basis for identifying the cytoplasm with the Donnan system: if *A* was 600 m.equiv./l. and the counterions monovalent, this phase would have an osmotic pressure of about 11 atmospheres, provided the activity of the counterions was similar to that in aqueous solution. The solution in the cytoplasm would then be under the same hydrostatic pressure as that in the vacuole since observations of the point of incipient plasmolysis show that the vacuolar O.P. is also about 11 atmospheres. The O.P. of the cytoplasm would, however, depend on the proportion of bivalent to univalent counterions in it, since the O.P. is a function of the molarity and not of the number of equivalents. The cytoplasm would be expected to change volume as this proportion changed, with a concomitant change in *A*. The experiments here reported were not precise enough to have detected this change had it occurred. The question of change of cytoplasmic volume is considered in more detail elsewhere by Briggs (1957).

If the Donnan phase is in the cytoplasm, then there appears to be no great resistance to isotopic exchange of cations between cytoplasm and the surrounding solution (Fig. 1*a*).

At present our knowledge of the free space is too limited to provide a full explanation of the difference between the value of *D* for Rb in water and that calculated on the assumption that the free space is uniform in composition, and in cross-sectional area in planes parallel to the surface of the disk.

ACKNOWLEDGEMENTS

One of us (A. B. H.) gratefully acknowledges receipt of an Australian Commonwealth Scientific and Industrial Research Organization Overseas Studentship, and another (M. G. P.) the receipt of an Agricultural Research Council Research Studentship.

LITERATURE CITED

BRIGGS, G. E., (1957). Some aspects of free space in plant tissue. *New Phytol.* **56**, 305–24.

—— and ROBERTSON, R. N. (1957). Apparent Free Space. *Ann. Rev. Plant Physiol.* **8**.

EPSTEIN, E. (1955). Passive permeation and active transport of ions in plant roots. *Plant Physiol.* **30**, 529–35.

—— (1956). Mineral nutrition of plants. Mechanisms of uptake and transport. *Ann. Rev. Plant Physiol.* **7**.

HOPE, A. B. (1953). Salt uptake by root tissue cytoplasm; the relation between uptake and external concentration. *Austrl. Journ. Biol. Sci.* **6**, 396–409.

ROBERTSON, R. N. (1951). Mechanisms of absorption and transport of inorganic nutrients in plants. *Ann. Rev. Plant Physiol.* **2**, 1–25.

STILES, W., and SKELDING, A. D. (1940). Salt relations of storage tissues. II. The absorption of manganese salts by storage tissues. *Ann. Bot.* N.S. **4**, 673–700.

SUTCLIFFE, J. F. (1952). The influence of internal ion concentration on Potassium accumulation and salt respiration of Red Beetroot tissue. *J. Exp. Bot.* **3**, 59–76.

TENDELOO, H. J. C., VERVELDE, G. J., and ZWART VOORSPUIJ, A. J. (1946). Electro chemical behaviour of ion-exchanging substances. II. Potential measurements on plant roots and their bearing on ion uptake by plants. *Rec. Trav. Chim.* **65**, 539–44.

The Organic Acid Metabolism of Bramley's Seedling Apple Peel[1]

G. E. NEAL AND A. C. HULME

Ditton Laboratory, Department of Scientific and Industrial Research

Received 1 July 1957

SUMMARY

1. The effect of various Krebs cycle acids on the respiration of disks of apple peel at various stages of maturity was measured in a Warburg respirometer.

2. Peel tissue from apples at the pre-climacteric and early post-climacteric stages apparently contain sufficient of the Krebs cycle acids used, with the exception of succinate, to maintain oxidative processes at a maximum.

3. The addition of malate causes a large increase in the CO_2-output of peel from post-climacteric and senescent fruit but not from pre-climacteric fruit, and a close correlation exists between the climacteric and this decarboxylation of malate. The decarboxylation of malate does not affect the rate of O_2-uptake of peel tissue. The possible part played by the decarboxylation of malate in the increased CO_2-output at the climacteric is discussed.

4. Added pyruvate is decarboxylated by the tissue at all stages of storage life.

5. The decarboxylation of added malate is an aerobic fermentation, resulting in the quantitative production of acetaldehyde. Although the presence of oxygen is necessary, the rate of O_2-uptake is not affected by the reaction. Pyruvate decarboxylation does not require the presence of oxygen.

6. The O_2-uptake of peel from senescent apples can be stimulated by addition of malate, succinate, and α-ketoglutarate. No evidence was obtained, however, of oxidation of fumarate, citrate, or pyruvate. The addition of malate to senescent tissue restores the lower endogenous rate of O_2-uptake to that of early post-climacteric tissue.

7. Succinate and fumarate are toxic to peel tissue at concentration above 0·02M.

INTRODUCTION

THE work described in this paper forms part of a study of the effect of organic acids on the respiration of apple tissue and of the acid metabolism of English varieties of apples. Porritt (1951) who investigated the effects of various organic acids on pulp tissue slices of Yellow Newton apples, found no effect on respiration on adding fumarate, and only a very slight increase on adding citrate. Lower concentrations of succinate were stimulatory, but higher concentrations proved to be inhibitory. The addition of pyruvate was without effect on O_2-uptake, but induced a large increase in CO_2-production. The addition of malate also produced a large increase in CO_2-production, but induced little change in O_2-uptake. He concluded that his results 'indicate participation of most of the acids of the Krebs cycle in CO_2-production, but do not necessarily demonstrate an organization such as a Krebs cycle'.

[1] The work described in this paper forms part of a Thesis submitted to London University.

Siegelman and Schomer (1954) used peel slices of Delicious, Red Delicious, and Grime's Golden apples in their investigation of the effect of scald (a physiological disease causing browning of peel) on apple peel respiration. They noted a large effect of added pyruvate on the CO_2-output of peel tissue, but not on O_2-uptake. This type of response was also produced by addition of boiled apple juice, which presumably contained a large quantity of malate. These effects of added organic acids on the respiration and the respiratory quotient (R.Q.) of apple tissue are interesting, and similar phenomena have been reported by workers using other plant-storage tissues, Bennet-Clark and Bexon (1943) using beetroot, Turner and Hanley (1949) using carrot, and Boswell (1945) using potato. No simple explanation of the effect in terms of a Krebs cycle mechanism is possible. Pearson and Robertson (1954) on the basis of results obtained with particles isolated from Granny Smith apples have, however, suggested that a Krebs cycle system operates in apples. In the present studies, peel-tissue slices were used rather than pulp slices, partly because of their higher metabolic activity (in agreement with the results of Hackney, 1946) and also because of their greater ease of handling. Peel from apples at several stages of maturity was used, since it was hoped that the results might throw some light on the storage behaviour of the fruits.

MATERIALS AND METHODS

Plant material. Locally grown unblemished Bramley's Seedling apples of medium size were used. The fruits were normally picked during the second or third week in September. With one exception experiments were confined to fruit from storage. For the purpose of this report, three stages of storage maturity are designated. Pre-climacteric, early post-climacteric (hereafter just called post-climacteric), and senescent. The term 'pre-climacteric' is used to describe the maturity of fruits which had not reached the onset of the climacteric rise in respiration. For these pre-climacteric experiments fruit was placed at 12° C. on picking. It was found that if these fruits were placed in cold (3–5° C.) storage directly on picking the peel subsequently behaved in the same way as peel from post-climacteric fruit. This finding was apparently connected with the observation that fruit placed in cold storage and subsequently removed to higher temperatures underwent the climacteric rise very rapidly, as compared with fruits which were picked at the same time and placed directly in a warmer store. Peel prepared from fruits placed directly in the warmer store on picking showed gas exchanges similar to peel tissue prepared from freshly picked fruit up to the onset of the climacteric. A bulk of apples was placed at 3° to 5° C. and used for the post-climacteric and senescent tissue experiments. The term 'post-climacteric' is used to describe the stage of maturity of fruit kept at 12° C. for about one month after the completion of the climacteric rise in respiration, during which time the fruits remained firm and fresh green in appearance and gave similar gas exchanges. It was found that peel from apples stored at 3° or 5° C. for 2 to 3 months, respired similarly to the post-climacteric tissue from apples at 12° C., although the fruits

underwent no definite climacteric at the lower temperature. Most of the post-climacteric tissue used in experiments was therefore prepared from apples in cold storage (3° to 5° C.). The term 'senescent' is used to describe the stage of maturity of fruit which had become soft and yellow in appearance but had not reached the stage of physiological breakdown. This senescent stage was reached after approximately 2 to 3 months at 12° C. and 6 to 7 months at 3° or 5° C. It must be pointed out that, although at 12° C. the pre- and post-climacteric stages of maturity are separated by a distinct phenomenon, the climacteric, the post-climacteric and senescent stages of maturity are two arbitrary stages of a continuous running down process.

Preparation of tissue slices. The method of preparing peel disks was based on that of Siegelman and Schomer (1954). All preparations were carried out at 1° C. The apples were peeled with a household potato peeler and the adhering cortical tissue removed from the peel strips by careful scraping with a porcelain spatula. This resulted in the preparation of peel tissue about 10 cells in depth, consisting of cuticle, epidermis, sub-epidermal chlorenchyma, and a part of a zone of cells transitional between peel and pulp. The good agreement between the respiration rates of peel tissue prepared on different occasions, and also microscopic examination, showed that a uniform tissue strip was prepared. Disks 1 cm. diameter were cut out of the strips with a stainless steel cork-borer.

Manometric technique. 1 g. of peel disks suspended in 3 ml. of 0·1M phosphate buffer pH = 4·0 were used in each Warburg flask. The direct method of Warburg was used. Experiments were carried out at least in duplicate. The molarity of added substrates, which had been brought to pH 4 with KOH, is given for the whole of the suspending medium. The respiration rate was allowed to reach a steady level before substrates were added. The temperature of the bath was 28° C. In view of the presence of chloroplasts in peel tissue, the Warburg bath was shaded: no errors due to photosynthesis were detected. The Warburg flasks were shaken at speed of 100 complete oscillations per minute. Readings were taken at 15-minute intervals. In anaerobic experiments air was swept from the Warburg vessels by a stream of nitrogen from a cylinder for 15 minutes, small traces of oxygen being removed from the nitrogen by passage over heated copper in silica tubes.

Peel tissue disks prepared from apples at the post-climacteric stage of maturity have a high initial rate of CO_2-evolution, which gradually decreases throughout the course of an experiment. This high CO_2-evolution can be reduced, and a steadier rate of CO_2-production achieved, if the disks are aerated overnight in 0·1M phosphate buffer pH = 4·0 at 1° C. This washing procedure was frequently used, and the peel tissue used in the experiments to be described are consequently termed 'washed' or 'unwashed'. In contrast to the results obtained from washing carrot (Turner and Hanley, 1949) or beet-root (Bennet-Clark and Bexon, 1943) tissue, no increase in respiration was ever found on washing apple peel tissue in either phosphate buffer or distilled water.

Chemical analyses. Organic acid determinations were carried out on tissue at the end of some of the respiration experiments. The tissue was killed by rapid immersion in hot 95 per cent. ethanol, followed by maceration in an M.S.E. tissue-disintegrator. The residue was removed by centrifugation, and was subsequently re-extracted with 85 per cent. alcohol followed by centrifugation. This process was continued until all colouring matter had been removed, when all organic acids had also been removed. Since in these experiments the utilization of added organic acid substrate was to be determined, no separation of the disks from the suspending medium was necessary. The subsequent procedure of decolouring the combined supernatants with charcoal, after evaporation of the alcohol, followed by the separation and determination of the organic acids by 'gradient elution' from a Dowex 1 ion exchange resin column, was carried out as described by Hulme and Wooltorton (1957).

Estimations of the total sugar content of the tissue were carried out at the same time as the organic acid estimations described above. The sugars were determined in the eluate and washings from the Dowex 1 ion exchange column. The Hanes's modification of the Somogyi copper method (described by Hulme, 1950) was used.

Aldehyde estimations were carried out using the method of Stotz (1943) as modified by Beevers (1952). In these experiments, 5 g. of peel tissue in 15 ml. of 0·1M phosphate buffer were placed in 100 ml. conical flasks, which were attached to the Warburg shaking apparatus so that they were almost completely immersed in the water. Substrates were added to some of the flasks at the beginning of the experiments. A stream of air was bubbled through the conical flasks and then passed through gas-washing bottles containing 100 ml. of 4 per cent. bisulphite solution, immersed in an ice bath. Under these conditions it was found that for complete removal of acetaldehyde at 280° C., an air flow of 10 litres/hr. was necessary. Aldehyde estimations were carried out on 1 ml. aliquots of the bisulphite solution, diluting if necessary. Since the Stotz colorimetric method is not specific for acetaldehyde, the identity of the aldehydes present in the bisulphite solution was always checked by the formation of 2:4-dinitrophenylhydrazones and subsequent paper partition chromatography (Meigh, 1956). In the experiments to be described, no significant amounts of aldehydes other than acetaldehyde were ever detected in the bisulphite solution. Initially, the quantitative chromatographic method of analysis of aldehydes (Meigh, 1956) was applied directly to vessels which had not been aerated by the method described above. Substantially the same results were obtained as with using the Stotz method, and as it was decided to use the more rapid Stotz method for the remainder of the work, CO_2-production by the tissue was estimated simultaneously with the aldehyde experiments described above. A CO_2-free air stream at 10 l./hour was passed through similar 100 ml. conical flasks containing 5 g. tissue and the CO_2 absorbed by soda was determined by titration.

Alcohol was estimated using 5 g. samples of tissue by the method of Williams and Reese (1950) as modified by Beevers (1952).

EXPERIMENTAL RESULTS

Effects of acids on peel tissue respiration. As a result of a preliminary survey using a range of concentrations of Krebs cycle acids, it was decided to use malate, pyruvate, α-ketoglutarate and citrate at a final concentration of 0·1M. This concentration either produced maximum stimulation or, in other cases where no stimulation occurred, higher concentrations were either equally ineffective or inhibitory. The specific responses of individual acids will be

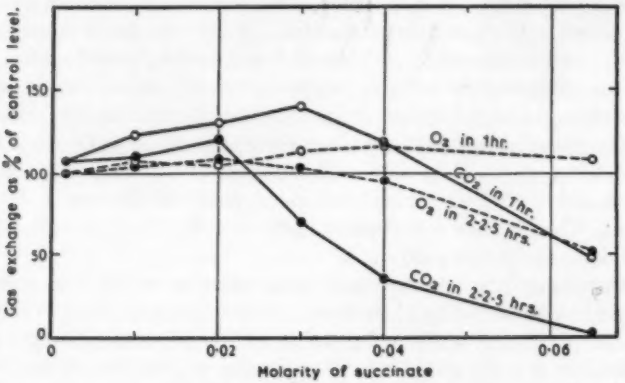


FIG. 1. The effect on the O_2-uptake and CO_2-output of peel disks from washed post-climacteric tissue treated with increasing concentrations of succinate.

referred to later. In the cases of succinate and fumarate, however, concentrations above 0·02M proved to be inhibitory, and above 0·05M, toxic (Fig. 1).

The inhibition affected the CO_2-output of the tissue to a greater extent than the O_2-uptake. A similar pattern of response was obtained with 'washed' or 'unwashed' tissue at all stages of storage.

The effects of the acids on the respiration rate of pre-climacteric tissue are shown in Fig. 2. The tissue was used directly on preparation in these experiments, as it was thought to be possible that washing might remove substrates from the tissue and give results not directly applicable to the intact peel.

Similar data for 'unwashed' post-climacteric and also senescent peel tissue are also given in Fig. 2.

Succinate alone stimulated O_2-uptake in pre-climacteric and post-climacteric tissue. Pyruvate addition gave a slightly larger stimulation in CO_2-output of post- than pre-climacteric tissue. Malate gave an even greater CO_2 effect. Clearly this effect of malate on the CO_2-production of the peel tissue had developed over the period of the climacteric rise in respiration in the whole fruit (see later).

Senescent tissue had a lower endogenous rate of O_2-uptake and CO_2-output than pre- or post-climacteric tissue (these two types of tissue having similar O_2-uptakes but different CO_2-outputs—to be referred to later). O_2-uptake

was approximately 60 per cent. of the post-climacteric level, and R.Q.s were only slightly in excess of unity using freshly prepared tissue disks and could be reduced to 1·0 on washing overnight. The stimulation of O_2-uptake on addition of succinate was increased, the final level attained being approximately that of succinate-stimulated post-climacteric tissue. Malate and α-ketoglutarate addition also stimulated O_2-uptake, and it was noticeable

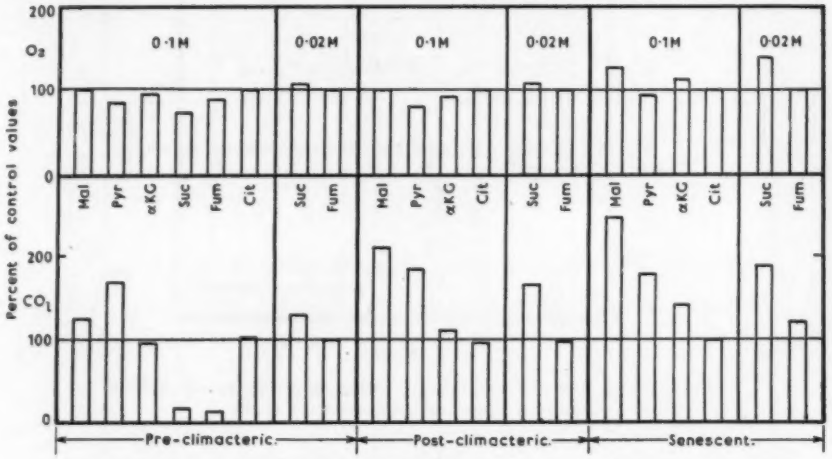


FIG. 2. The effects on the O_2-uptake and CO_2-output of adding various acids to respiring disks of pre- and post-climacteric and senescent peel, expressed as a percentage of the values obtained for disks to which no acid had been added (control disks). The values are those obtained for the period ½–1½ hours after the additions.

that malate addition invariably resulted in a final level of O_2-uptake similar to the endogenous level of post-climacteric tissue, Fig. 3.

The pattern of effects of addition of the organic acids on CO_2-output of senescent peel was similar to that of post-climacteric tissue, except that fumarate resulted in a small increase for the first time. The higher percentage increases in CO_2-production induced by malate, pyruvate, and succinate and α-ketoglutarate were due to the lower endogenous level of CO_2-uptake, and the stimulated levels were usually slightly lower than the post-climacteric stimulated levels. It is noteworthy that at no stage did an addition of citrate result in a stimulation of O_2-uptake or CO_2-output, and fumarate and pyruvate were without effect on the level of O_2-uptake.

It appeared likely that the oxidative responses produced by addition of certain acids to senescent peel tissue were due to acid-substrate exhaustion. This suggestion is supported by the lower rate of endogenous respiration and the lower R.Q. values for this tissue. Experiments were therefore carried out in which senescent tissue was aerated in distilled water at 1° C. for 90 hours, in an attempt to increase substrate exhaustion. No contamination from micro-

organisms was evident during this procedure, the air being passed through a fine Seitz filter before passing through the washing flasks. This washing process reduced the endogenous level of O_2-output to 36 per cent. of that of post-climacteric tissue. It was necessary to add all the acids at 0·02M concentration to this type of tissue, since the higher concentrations previously

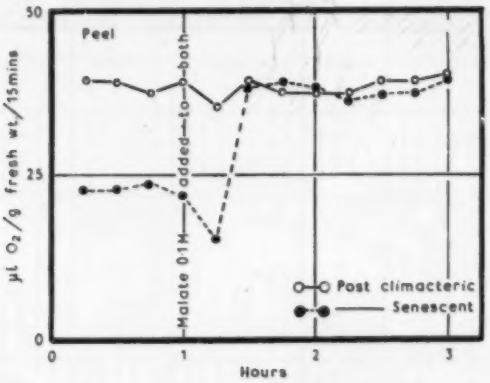


FIG. 3. The effect of the addition of malate on the O_2-uptake of disks from post-climacteric and senescent apples.

employed resulted in temporary inhibition, presumably due to a higher rate of penetration into the softer tissue. The results are shown in Fig. 4.

The same pattern of response to the added acids was obtained as with unwashed senescent tissue, although succinate, while producing the largest stimulation, only restored the level of O_2-uptake to 83 per cent. of the endogenous post-climacteric level, compared with 112 per cent. when using freshly prepared senescent tissue. Washing had therefore either lowered the efficiency of the oxidative enzyme systems utilizing the acids, or other oxygen-requiring processes had also become depleted. Again no oxidative utilization of citrate, pyruvate, or fumarate was demonstrated.

Malate and pyruvate decarboxylation by peel tissue. The decarboxylation reactions utilizing malate and pyruvate, mentioned above, are interesting since increased oxygen uptake was not found. This is surprising in the case of malate, since the primary utilization of this acid might be expected to involve malic dehydrogenase and hence an increased O_2-uptake. Experiments were carried out to determine if the malate and pyruvate decarboxylation occurred in absence of O_2.

Post-climacteric tissue washed overnight at 1° C. was used, and the data given in Fig. 5 show that, whereas the pyruvate decarboxylation was capable of proceeding under anaerobic conditions, malate decarboxylation needed the presence of O_2, even though the overall O_2-uptake was unaffected. Fig. 5 also shows that O_2 was not merely necessary for the initiation of the malate

decarboxylation reaction, since when tissue in which the reaction was proceeding was later placed in nitrogen, the high CO_2-output was almost completely suppressed. A further point illustrated by Fig. 5 is the sustained high level of CO_2-production induced by malate-addition compared with the decline from an initial peak level induced by pyruvate.

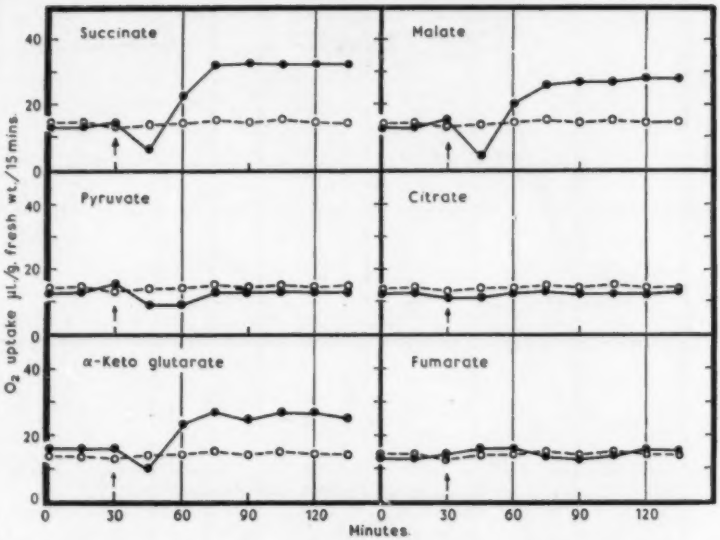


FIG. 4. The effect on the O_2-uptake of adding various acids to peel disks from senescent apples which had been previously aerated in distilled water for 90 hours at 1° C.

It appeared possible from the initial experiments that the malate decarboxylation reaction was a means of utilizing malate, in excess of oxidative requirements, mainly by post-climacteric and senescent peel tissue. In order to test this malate at a high and a low concentration was added to senescent tissue which had been washed overnight at 1° C. in phosphate buffer. Malate stimulates O_2 and CO_2 exchanges of senescent tissue.

Data of Fig. 6 show that both concentrations of malate restored the level of O_2-uptake to the endogenous level of post-climacteric tissue, but the effect of 0·01M malate on CO_2-production was only half that of 0·1M malate. Clearly added malate at the senescent stage was preferentially utilized in a process involving O_2-uptake.

The mechanism of the decarboxylation of malate. There are two likely possibilities; either added malate might be utilized to form sugars, via a reversal of the glycolytic pathway (Bennet-Clark and Bexon, 1943) or it might be aerobically fermented (Turner and Hanley, 1949). Aldehyde, sugar, malate utilization, and CO_2-production determinations were, therefore, carried out

on malate-treated and control tissues at the end of a Warburg experiment. The results of a typical experiment are given in Table I, in which the amount of tissue used was 5 g. and the experimental period was 7·5 hours.

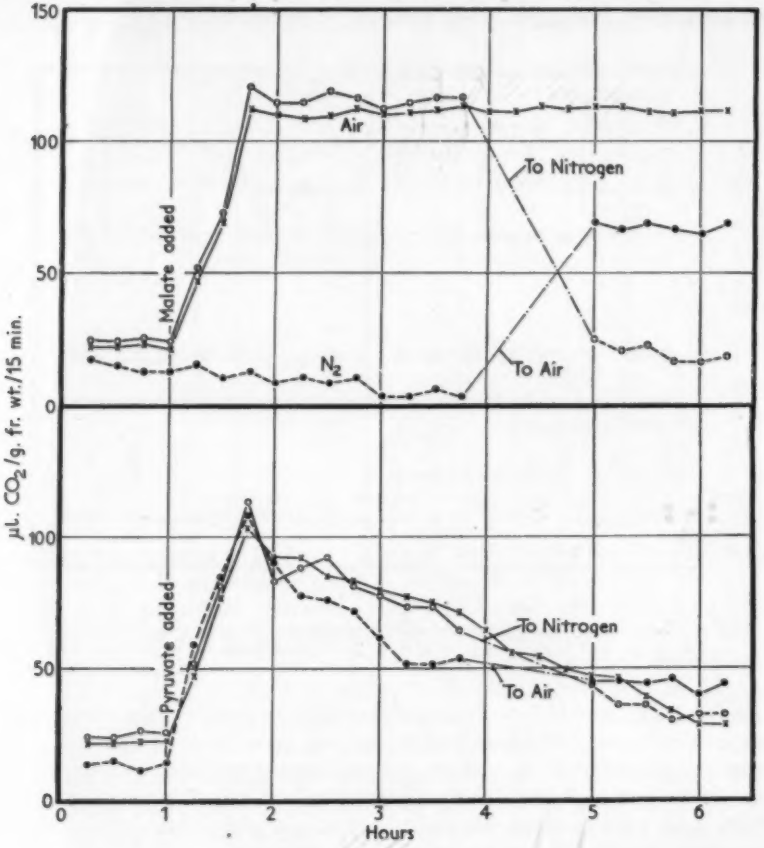


FIG. 5. The effect of anaerobic conditions (broken lines) and aerobic (continuous lines) on the decarboxylation of malate (upper graph) and pyruvate (lower graph) at 0·1M concentration (external) by apple peel disks from post-climacteric apples.

The low malate content of the tissue used in this experiment was due, presumably, to the fact that apples were nearing the end of the post-climacteric stage; the tissue was further depleted by the overnight washing. No significant difference between the sugar content of control and malate-treated tissue was found. Acetaldehyde was produced by the malate-treated tissue, the ratio CO_2 produced/acetaldehyde formed/malate utilized was close to the theoretical value of 2/1/1. This type of experiment was carried out on several occasions,

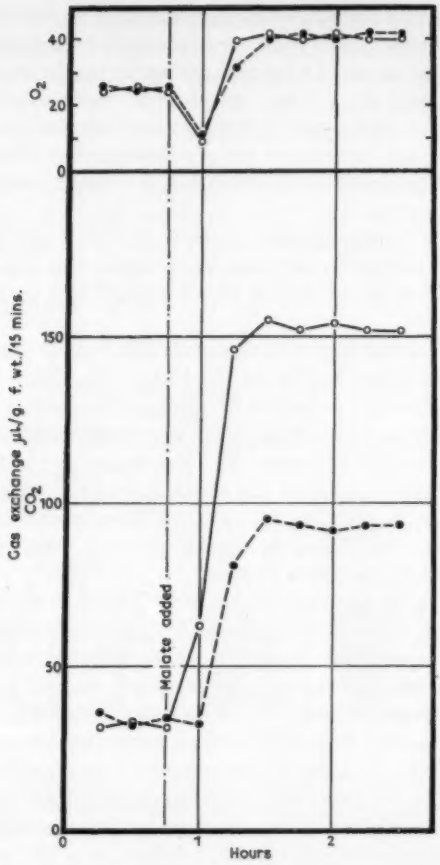


FIG. 6. The effect of adding malate at two concentrations on the O_2-uptake and CO_2-output of peel disks from senescent apples.

TABLE I

	CO_2 produced (ml.)	Acetaldehyde formed (mg.)	Malic acid content (mg.)	Total sugar content (mg.)
Initial	—	—	3·2	41·2
Final control	2·45	0·017	0·8	40·1
Final malate added 189 mg.	8·01	5·60	172·5	39·9
Difference in ml. and mg.	5·56	5·58	15·7	—
Difference in μ mols.	248	127	117	—

and in some cases the acetaldehyde was estimated by the chromatographic method of Meigh (1956), in all these experiments the same ratio was obtained. Alcohol determinations, carried out as described in the Experimental Methods Section, showed very slight amounts in both malate-treated and control tissue, there being no significant differences between the two.

Similar experiments carried out on pyruvate-treated tissue again showed that acetaldehyde accumulated. The ratio CO_2-produced/acetaldehyde formed in these experiments was 1/1.

Determinations of acid contents showed that after the addition of acid substrates the concentration of these acids *inside* the disks of tissue was approximately one-tenth of that of the concentration in the surrounding medium.

Malate decarboxylation by peel tissue and the climacteric. In the initial experiments the effect of added malate on the CO_2-production of peel tissue increased strikingly over the period in which the climacteric rise in respiration was taking place in the whole fruit. Most explanations advanced to account for the climacteric in fruits (Pearson and Robertson, 1954; Millerd, Bonner, and Biale, 1953) have suggested the formation in the tissue of substances which effect 'uncoupling' of phosphorylations from oxidations. The apparent correlation between the climacteric and a metabolic reaction of a different kind was, therefore, further investigated.

Apples, picked on 13 September 1954, were placed in single apple-respiration chambers at 12° C. immediately on picking, and CO_2 outputs are recorded in the upper portion of Fig. 7. At various stages, indicated by the open circles, apples were removed from the respiration chambers and peel disks quickly prepared at 1° C. Respiration of these disks was determined for 2 hours in the Warburg apparatus, and then malate (final concentration 0·1M) was added. The increase in CO_2 production was measured over the period from 1 to 2 hours after malate addition. Both malate-stimulated and endogenous rates of CO_2-production are given in Fig. 7. It can be seen that there was a gradual increase in the endogenous rate of CO_2-production of the peel disks during the course of the experiment, and a considerable increase in the malate stimulation. This increase in malate stimulation is closely correlated with the climacteric in the whole apple from which the disks were prepared. There was a small effect of malate on the CO_2-production of peel from the apples used earliest. However, it had been found that the CO_2-output of peel from apples picked a fortnight earlier was not stimulated by malate. It appeared, therefore, that the 'malate-effect' appeared slightly in advance of the climacteric in the whole apple, but did not develop rapidly until the onset of the climacteric.

Also given in Fig. 7, in circles in the appropriate histograms, are the R.Q.s of the endogenous respiration rate of the peel disks during the first 2 hours of the Warburg experimental periods. It can be seen that these increased throughout the course of the experiment from 1·4 to 1·9 which was due entirely to the increase in endogenous CO_2-production, there being no change in

O_2-uptake. As stated earlier, it was possible to reduce this high CO_2-production level by washing the disks overnight, but it was not possible to reduce the R.Q.s to unity, in contrast with the results obtained with senescent tissue. The peel from apples picked a fortnight earlier than those used in Fig. 6, which had no 'malate-effect', also had R.Q.s of approximately unity.

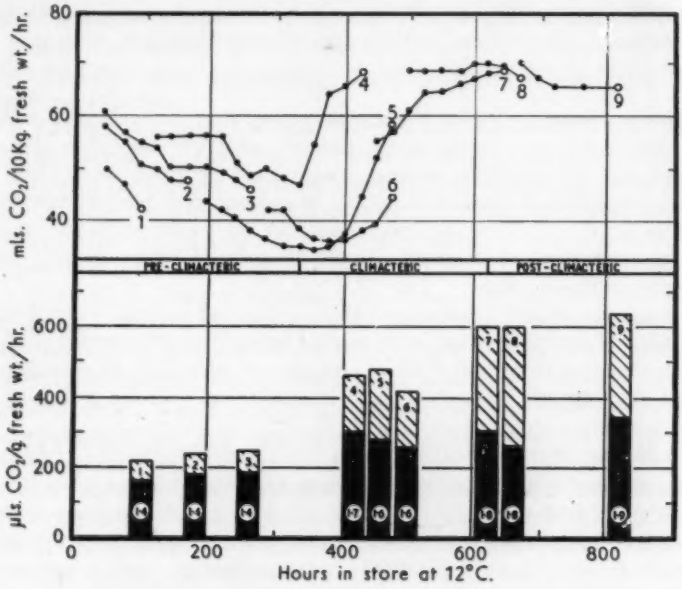


FIG. 7. The effect of adding malate (0·1M) to the peel disks prepared from apples at various stages during the development of the respiration climacteric in the whole fruit. The respiration of the apples from which the peel disks were prepared are shown in the upper graph. The figures attached to the 'curves' in the upper graph correspond with the histograms of CO_2-output shown in the lower graph. The black areas in the histograms give CO_2-output of disks before addition of malate and shaded areas show extra output after this addition.

In other experiments it was found that the 'pyruvate-effect' on the CO_2-production of peel tissue did not alter appreciably over the time of the climacteric rise in respiration in the whole fruit.

DISCUSSION

The fact that it was not possible to stimulate the rate of O_2-uptake of pre- or post-climacteric tissue by addition of any of the acids used (except succinate) but that stimulation of senescent peel tissue occurred with malate and α-keto glutarate (and an increased stimulation with succinate) would appear to indicate that senescent peel tissue is deficient in organic acid substrates. Peel tissue from apples at the senescent stage of maturity would therefore appear to be the most suitable material for the examination of the possible operation

of a Krebs cycle mechanism. However, the O_2-uptake of this tissue was not stimulated by addition of fumarate, pyruvate, or citrate. Qualitative evidence has since been obtained that citrate does penetrate into the tissue disks. The results obtained from addition of fumarate are particularly surprising, since its effect would be expected to be intermediate between those of malate and succinate but with neither O_2-uptake nor CO_2-output was this found to be so. Results obtained by Allentoff, Phillips, and Johnston (1954) using $^{14}CO_2$ to study fixation in Bramley's Seedling apples, whilst indicating that the

$$\text{Fumarate} \rightleftharpoons \text{Malate} \rightleftharpoons \text{Oxaloacetate} \rightleftharpoons \text{Pyruvate}$$

system was operating in the tissues, suggested that the part of the Krebs cycle concerned with citrate and aconitate, *iso*citrate was either absent or operated only slowly. This finding is supported by other work (Neal and Hulme, unpublished results) in which it was not possible to demonstrate aconitase activity. It must, however, be pointed out that citrate is a normal, if minor component of Bramley's Seedling apples. Further work is clearly necessary on these points.

The capacity of peel tissue to decarboxylate added malate, which develops at the time of the climacteric, is an aerobic fermentation process resulting in the accumulation of acetaldehyde. The observed ratio CO_2 produced/malate utilized/acetaldehyde formed, 2/1/1, is in agreement with the pathway

$$\text{Malate} \xrightarrow{-2H} \text{Pyruvate} + CO_2 \longrightarrow \text{Acetaldehyde} + CO_2.$$

The existence of this system is further supported by preliminary results using the method of Isherwood and Niavis (1956) to determine pyruvic acid. Small accumulations of pyruvate occur in malate treated tissue; these accumulations are much greater if sodium fluoride is added at the same time as malate. The fact that the O_2-uptake of the tissue did not alter when malate was added indicates that other oxygen requiring processes proceeding in the tissue must be correspondingly reduced. The participation of oxygen in the reaction was indicated by the results of the anaerobic experiments.

This decarboxylation reaction probably accounts for the high R.Q.s encountered on cutting apple tissue, the 'wound effect' being due to malate released from damaged cells. This would also account for the fact that the high R.Q.s could be reduced by washing the tissue, resulting in a removal of the released juice. It also probably accounts for the stimulation in CO_2-output but not O_2-uptake obtained by Siegelman and Schomer (1954) on adding boiled apple-juice to peel tissue slices. Although it was not possible to demonstrate the decarboxylation reaction under anaerobic conditions using tissue disks, it may well have some bearing on the observation of Fidler (1951) that acid loss in whole apples can be the same in nitrogen as in air. He also found that some mechanism must exist for the fermentation of malic acid both in air and nitrogen.

Acetaldehyde is toxic to apple tissue at low concentrations (Thomas, 1925). If the malate decarboxylation reaction is a feature of the normal metabolism

of Bramley's Seedling apples in storage, then some mechanism must exist for the removal of acetaldehyde formed, other than conversion to alcohol. It has been found that if acetaldehyde is not removed from tissue disks (by aeration) when malate is added, a quantitative relationship is no longer obtained between CO_2 produced and acetaldehyde formed. Furthermore, in view of its toxic nature, acetaldehyde must be metabolized in some way in view of the sustained nature of the malate-stimulated CO_2-production of apple peel (up to 6 hours). Fidler (unpublished results) has indeed obtained evidence for the metabolism of acetaldehyde by Bramley's Seedling apples other than by conversion to alcohol.

The fact that the malate-decarboxylation reaction becomes active at the time of the climacteric may well have some bearing on the climacteric itself. Hulme (1954) has suggested that the increase in protein in apples at the time of the climacteric might be associated with the formation of new enzymes. Tager and Biale (1957), observing that carboxylase activity develops during the climacteric rise in bananas, have suggested that the synthesis of carboxylase is in keeping with this increase in protein. The present results for the apple, however, suggest that the system formed during the climacteric is the one responsible for the decarboxylation of malate to pyruvate. Decarboxylation of pyruvate (carboxylase) proceeds at the same rate in both pre- and post-climacteric apples. There is nothing surprising in these differences. Change in the 'enzyme-pattern' is in keeping with the concept of a profound modification of the metabolism of fruits as they pass from 'growth' to 'ripening'. It is now becoming clear that in different fruits, during their whole life history, different metabolic processes predominate. It is therefore to be expected that dissimilar changes will occur in different kinds of fruit during the development of the ripening processes.

Little is known concerning R.Q.s of whole apples over the climacteric, but it is suggested that part at least of the extra CO_2-produced at the time of the climacteric may well come from a malate-decarboxylation reaction. It is suggested that malate is utilized prior to the climacteric in processes involving direct oxidation which yield an R.Q. close to unity, but that during the development of the climacteric an additional purely decarboxylative process, in which aldehyde is formed via pyruvate, either commences *de novo* or is greatly augmented. The R.Q. values of the tissue slices support this theory, since although the R.Q.s of early pre-climacteric and senescent peel tissue is approximately unity, it is not possible to reduce the R.Q. of early post-climacteric to this level, even after prolonged washing to remove the external 'juice-effect'.

The toxic effect of concentrations of succinate and fumarate higher than 0·02M on apple peel tissue (and also pulp tissue—Neal and Hulme, unpublished results) probably has considerable bearing on the recent finding that succinic acid accumulated in Bramley's Seedling apples showing symptoms of incipient CO_2 injury (Hulme, 1956). It should be remembered (p. 152) that the concentration of acid inside the tissue is only about one-tenth of that in

the outside solution. Toxicity of succinate towards carrot tissue has been reported by Turner and Hanley (1949) who ascribed it to an effect on the cell membrane. Succinate alone out of the organic acids used in this study was capable of increasing the rate of O_2-uptake of pre- and post-climacteric peel tissue. This permanent unsaturation of enzyme systems capable of oxidizing succinate would presumably be correlated with the fact that succinate does not normally occur free in these apples. Addition of low concentrations of succinate, as well as stimulating O_2-uptake, also increased the rate of CO_2-output of the tissue, particularly at the post-climacteric and senescent stages of maturity. Quantitative experiments showed that the increased CO_2-output induced by addition of succinate also resulted in an accumulation of acetaldehyde. This suggested that the succinate was first converted to malate (accounting for the increased O_2-uptake at all stages of maturity) and that after the climacteric the malate formed could be decarboxylated to give CO_2 and acetaldehyde (cf. malate effect). The toxic action of higher concentrations of fumarate may be a result of its conversion to succinate. Qualitative analysis showed that when fumarate was added to post-climacteric tissue, succinic acid subsequently appeared in the tissue. These results suggest that although free succinic acid is not a normal constituent of the fruit, it may nevertheless be a normal metabolite.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

LITERATURE CITED

ALLENTOFF, N., PHILLIPS, W. R., and JOHNSTON, F. B. (1954). ^{14}C study of carbon dioxide fixation in the apple. II. Rates of carbon dioxide fixation in the detached McIntosh apple. *J. Sci. Food Agric.* **5**, 234.

BEEVERS, H. (1952). Malonic acid as an inhibitor of maize root respiration. *Plant Physiol.* **27**, 725.

BENNET-CLARK, T. A., and BEXON, D. (1943). Water relations of plant cells. III. Respiration of plasmolysed tissues. *New Phytol.* **42**, 65.

BOSWELL, J. G. (1945). Oxidation systems in the potato tuber. *Ann. Bot.* N.S. **9**, 55.

BURRIS, R. H. (1949). *Manometric techniques and tissue metabolism.* Burgess Publishing Co. Minn.

FIDLER, J. C. (1951). A comparison of the aerobic and anaerobic respiration of apples. *J. Exp. Bot.* **2**, 41.

HACKNEY, FRANCES M. V. (1946). Studies in the metabolism of apples. VI. Preliminary investigations on the respiration of sliced apple tissue. *Proc. Linnean Soc. N.S.W.* **70**, 333.

HULME, A. C. (1950). Data for the study of the metabolism of apples during growth and storage. Section II. *J. Hort. Sci.* **25**, 277.

—— (1954). Studies in the nitrogen metabolism of apple fruits. The climacteric rise in respiration in relation to changes in the equilibrium between protein synthesis and breakdown. *J. Exp. Bot.* **5**, 159.

—— (1956). Carbon dioxide injury and the presence of succinic acid in apples. *Nature*, **178**, 218.

—— and WOOLTORTON, L. S. C. (1958). A comprehensive scheme for the determination and isolation of the non-volatile organic acids of pome fruits, and its application to a study of changes in the individual acids in detached apples during a storage at 15°C. *J. Sci. Food Agric.* (In the press).

ISHERWOOD, F. A., and NIAVIS, C. A. (1956). Estimation of α-keto acids in plant tissue, a critical study of various methods of extraction as applied to strawberry leaves, washed potato slices, and peas. *Biochem. J.* **64**, 549.

MEIGH, D. F. (1956). Volatile compounds produced by apples. 1. Aldehydes and Ketones. *J. Sci. Food Agric.* **6**, 396.

MILLERD, A., BONNER, J., and BIALE, J. B. (1953). The climacteric rise in fruit respiration as controlled by phosphorylative coupling. *Plant. Physiol.* **28**, 521.

PEARSON, J. A., and ROBERTSON, R. N. (1954). The physiology of growth in apple fruits. VI. The control of respiration rate and synthesis. *Aust. J. Biol. Sci.* **7**, 1.

PORRIT, S. W. (1951). *Production of carbon dioxide by apple tissue.* Annual report of Fruit and Vegetable Products Research Committee of Canadian Department of Agriculture.

SIEGELMAN, H. W., and SCHOMER, H. A. (1954). Effect of scald on apple skin respiration. *Plant Physiol.* **29**, 429.

STOTZ, E. (1943). A colorimetric determination of acetaldehyde in blood. *J. Biol. Chem.* **148**, 585.

TAGER, J. M., and BIALE, J. B. (1957). Carboxylase and aldolase activity in ripening banana. *Physiol. Plant.* **10**, 79.

THOMAS, M. (1925). The controlling influence of carbon dioxide. V. A quantitative study of the production of ethyl alcohol and acetaldehyde by cells of the higher plants in relation to concentration of oxygen and carbon dioxide. *Biochem. J.* **19**, 927.

TURNER, J. S., and HANLEY, V. F. (1949). Succinate and plant respiration. *New Phytol.* **48**, 149.

WILLIAMS, M. B., and REESE, H. D. (1950). Colorimetric determination of ethyl alcohol. *Anal. Chem.* **22**, 1556.

BOTANICAL BOOKS

New, Secondhand

Old and Rare

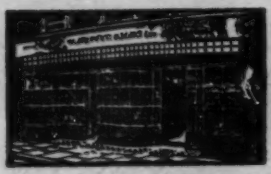
WHELDON & WESLEY
LTD.

LYTTON LODGE
Codicote, Near Hitchin
Herts.

Telephone: CODICOTE 370

Libraries and small parcels of books purchased for cash

HEFFER'S



BOOKS ON BOTANY IN ALL LANGUAGES

★

Catalogues available free; books and learned journals bought

★

W. HEFFER & SONS LTD.
Petty Cury · Cambridge

BOOKS ON BOTANY & ALLIED SCIENCES

Large Stock of Recent Editions

Continental and American books obtained to order with the least possible delay.

SECONDHAND DEPT.

Select stock of recent editions of books on Botany and Allied Sciences available. Back numbers of scientific journals obtainable. Books sought for and reported free of charge. Large or small collections bought.

LENDING LIBRARY

Scientific and Technical

Annual Subscription from £1. 17s. 6d.

Prospectus post free on request.

The library covers a wide range of subjects and is invaluable to students, and Research workers.

THE LIBRARY CATALOGUE, revised to December 1956, containing a classified Index of Authors and Subjects. To Subscribers, £1. 5s. net; to Non-Subscribers, £2. 2s. net. Postage 2s.

Bi-monthly List of New Books and New Editions sent post free to subscribers on request.

H. K. LEWIS & Co. Ltd.
136 GOWER STREET, LONDON, W.C.1
Telephone: EUSton 4282

Printed in Great Britain at the University Press, Oxford, by Charles Batey, Printer to the University